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Thyroid hormone receptors are tumor suppressors in a mouse model of metastatic follicular thyroid carcinoma

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Abstract

Aberrant expression and mutations of thyroid hormone receptor genes (TRs) are closely associated with several types of human cancers. To test the hypothesis that TRs could function as tumor suppressors, we took advantage of mice with deletion of all functional TRs (*TRα1*$^{-/-}$ *TRβ*$^{-/-}$mice). As these mice aged, they spontaneously developed follicular thyroid carcinoma with pathological progression from hyperplasia to capsular invasion, vascular invasion, anaplasia and metastasis to the lung, similar to human thyroid cancer. Detailed molecular analysis revealed that known tumor promoters such as pituitary tumor-transforming gene were activated and tumor suppressors such as peroxisome proliferator-activated receptor γ and p53 were suppressed during carcinogenesis. In addition, consistent with the human cancer, AKT–mTOR–p70^{S6K} signaling and vascular growth factor and its receptor were activated to facilitate tumor progression. This report presents *in vivo* evidence that functional loss of both *TRα1* and *TRβ* genes promotes tumor development and metastasis. Thus, TRs could function as tumor suppressors in a mouse model of metastatic follicular thyroid cancer.

Keywords

thyroid cancer; mouse model; mutations of thyroid hormone receptors

Introduction

Thyroid hormone nuclear receptors (TRs) are ligand-dependent transcription factors critical for growth, development and differentiation. Alternative splicing of the primary transcripts of the two TR genes, α and β, yields three major thyroid hormone (T3) binding isoforms: α1, β1 and β2. TRs regulate expression of target genes through their interaction with thyroid hormone response elements located in the promoter regions. Genes regulated by TRs include growth factors, cell surface receptors, transcription factors, cell-cycle regulators, oncogenes and tumor suppressors (Puzianowska-Kuznicka *et al*., 2006). Recently, it was also shown that TRs directly modulate the activities of signaling through protein-protein interaction with key effectors in the pathways (Davis *et al*., 2008; Guigon *et al*., 2008; Furuya *et al*., 2009). For example, TRs physically interact with the regulatory p85α subunit of phosphatidylinositol 3-kinase (PI3K) to modulate the downstream AKT-mammalian target

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of rapamycin (mTOR) and $p70^{S6K}$ and PI3K–integrin-linked kinase (ILK)–matrix metalloproteinase (MMP)-2 signaling pathways (Furuya *et al.*, 2009).

Early evidence suggesting that mutated TR could be involved in carcinogenesis came from the discovery that TRα1 is the cellular counterpart of the retroviral v-*erb*A that induces acute erythroleukemia and sarcomas in birds (Sap *et al.*, 1986; Weinberger *et al.*, 1986; Thormeyer and Baniahmad, 1999). v-*erb*A is a highly mutated chicken TRα1 that does not bind T3 and loses the ability to activate gene transcription. It competes with TR for binding to thyroid hormone response elements and interferes with the transcriptional activity of liganded TR on several promoters (Chen and Privalsky, 1993; Yen *et al.*, 1994). Since those early studies, mutated TRs have been reported to associate with several human cancers, including liver (Lin *et al.*, 1999), kidney (Kamiya *et al.*, 2002), pituitary (Safer *et al.*, 2001; Ando *et al.*, 2001a, b) and thyroid (Puzianowska-Kuznicka *et al.*, 2002). Reduced expression of TRβ1 mRNA was also implicated in the carcinogenesis of human kidney cancer (Puzianowska-Kuznicka *et al.*, 2000) and papillary thyroid carcinomas (Puzianowska-Kuznicka *et al.*, 2002; Takano *et al.*, 2003). The silencing of the TRβ gene by hypermethylation and the concurrent reduction of *TRβ1* transcripts were shown in breast cancer (Li *et al.*, 2002). In cotransfection experiments, it was shown that both TRα1 and TRβ1 strongly repressed Ha-$ras^{val\ 12}$-induced transformation of NIH3T3 fibroblasts, reduced tumor volume and inhibited tumor growth in nude mice (Garcia-Silva and Aranda, 2004). Recently, it was shown that the transfected *TRβ1* in hepatocarcinoma and breast cancer cells reduced tumor growth, caused partial mesenchymal-to-epithelial cell transition, and had a striking inhibitory effect on invasiveness, extravasation and metastasis formation in mice (Martinez-Iglesias *et al.*, 2009).

Previously, we created a knockin mutant mouse (*TRβPV* mouse) by targeting a mutation (denoted PV) to the *TRβ* gene locus through homologous recombination and the *Cre-LoxP* system (Kaneshige *et al.*, 2000). *TRßPV* was derived from a patient, PV, with a genetic disease known as resistance to thyroid hormone (Weiss and Refetoff, 2000). *PV* has a C-insertion at codon 448, which produces a frame shift of the COOH-terminal 14 amino acids of TRß1. PV has lost T3 binding completely and shows potent dominant negative activity (Meier *et al.*, 1992). As $TR\beta^{PV/PV}$ mice age, they spontaneously develop follicular thyroid carcinomas with a pathological progression similar to human thyroid cancer (Suzuki *et al.*, 2002; Ying *et al.*, 2003a, b). In subsequent studies, we found that mice with one mutated *TRβ* allele in the absence of the other wild-type allele ($TR\beta^{PV/-}$ mouse) also develop follicular thyroid carcinoma. The pathological progression of $TR\beta^{PV/-}$ mice is indistinguishable from that of $TR\beta^{PV/PV}$mice. Moreover, there is a striking similarity in the patterns of several altered signaling pathways between $TR\beta^{PV/-}$ mice and $TR\beta^{PV/PV}$ mice during carcinogenesis. Thus, the mutation of one *TRβ* allele in the absence of the other wild-type allele is sufficient to induce thyroid carcinoma. These findings indicate that the mutations of two *TRβ* alleles, as well as mutation of one *TRβ* allele in the absence of the other wild-type allele, lead to the development of metastatic follicular thyroid carcinoma. However, it is not clear whether the loss-of-function or gain-of-function mutation causes carcinogenesis. One possibility is that the mutant *TRβPV* could act as an oncogene through gain-of-function to promote carcinogenesis. Indeed, it was shown that PV could act through the gain-of-function mode to activate PI3K signaling pathways through physical interaction with the regulatory p85α subunit of PI3K whereas the wild-type TRβ1 has very weak activity (Furuya *et al.*, 2009). Alternatively, PV could, through the loss-of-function mode, act as a dominant negative mutant through the nucleus-initiated transcription to promote thyroid carcinogenesis (Kaneshige *et al.*, 2000; Zhang *et al.*, 2002; Suzuki *et al.*, 2003). Indeed, studies in $TR\beta^{PV/-}$ mice and $TR\beta^{PV/PV}$ mice suggest that PV could act through gain-of-function as well as a loss-of-function mutation (Kato *et al.*, 2006; Guigon *et al.*,

2008; Furuya *et al.*, 2009). However, it is not clear whether total loss of the normal functions of TRs alone is sufficient to lead to thyroid carcinogenesis.

To address this question, we took advantage of available mice that are devoid of all known functional TRs. Remarkably, we found that the $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice spontaneously developed follicular thyroid cancer as they aged. Histological evaluation showed the progression from hyperplasia to capsular invasion, vascular invasion, anaplasia and metastasis to the lung. Molecular studies revealed that multiple signaling pathways were altered to promote thyroid carcinogenesis. This study provided direct evidence to indicate that TRs could function *in vivo* as suppressors of follicular thyroid carcinomas.

Results

Spontaneous development of follicular thyroid carcinoma in TRα1$^{-/-}$ TRβ$^{-/-}$ mice

Figure 1 shows the Kaplan-Meier cumulative survival curves for $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice over 20.9 months. The 50% survival age was 9.3 months ($n = 94$). In contrast, wild-type $TR\alpha 1^{+/+}TR\beta^{+/+}$ mice (WT-mice) were healthy with no deaths during the same observation period.

Detailed characterization was carried out for moribund $>TR\alpha 1^{-/-}TR\beta^{-/-}$ mice. A common obvious abnormality was the marked enlargement of thyroid glands. The thyroid weights of $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice were 27.2 ± 2.9 (mean ± s.e.: $n = 6$), 56.7 ± 4.6 ($n = 10$) and 115 ± 27.1 ($n = 8$) mg at the ages of 2–5, 6–9 and 10–13 months, respectively. The average weight of adult thyroids of the wild-type ($TR\alpha 1^{+/+}TR\beta^{+/+}$) mice was 4.0 ± 0.3 mg ($n = 14$) and no significant changes in the thyroid weights of adult wild-type mice were observed as they aged. Thus, this represents a 7-, 14- and 29-fold enlargement in the thyroid glands of the $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice at the ages of 2−5, 6−9 and 10−13 months, respectively. The enlargement of thyroid glands was progressive as indicated by the increasing ratios of thyroid to body weight from 11.3 ± 1.1 at the age of 2–5 months to 36.8 ± 7.4 at the age of 10–13 months (Figure 2).

Histopathological evaluation of the thyroids of $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice showed grossly enlarged glands, displaying diffuse adenomatous hyperplasia with dense nuclear chromatin and a glandular pattern characteristic of follicular carcinoma. In the 6- to 12-month-old moribund mice, histopathological changes consistent with neoplastic progression of invasion of the thyroid capsule by follicular elements (Figure 3a), invasion of vascular spaces within and adjacent to the thyroid (Figure 3b), focal anaplasia of epithelial cells within the hyperplastic areas (Figure 3c) and finally appearance of distant metastatic lesions in the lung (Figure 3d). The metastatic lesions in the lung showed characteristics of both spindle cell anaplastic and follicular morphology, including focal accumulations of colloid. The nuclear features observed in papillary carcinoma of the thyroid in humans were not observed. This interpretation of histologic type is further supported by a selective pattern of lung metastasis, with no evidence of lymph node involvement, features characteristic of follicular carcinoma of the thyroid in humans. In contrast, the histological patterns of the thyroid glands from 10- to 12-month-old WT-mice showed no detectable hyper-plasia. The quantitative analysis showed progression of these pathological changes as the mice aged. At 2–5 months of age, only one-third of mice (two out of six) were observed to have capsular invasion. At the ages of 10–13 months, 85.7% (12 out of 14) of mice had capsular invasion (Figure 4a). Vascular invasion was not observed in mice younger than 5 months, but was observed only in mice approximately 15% (2 out of 13) aged 6–9 months, and in 35.7% (5 out of 14) aged 10–13 months (Figure 4b). Anaplasia was observed in mice only at the age of 10–13 months (Figure 4c). Approximately, 21% (3 out of 14) of mice had distant lung metastasis at the age

of 10–13 months (Figure 4d). This pathological progression is similar to human follicular thyroid carcinoma.

Alteration of growth and survival signaling pathways in thyroids of *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice

Along with others, we have shown that serum TSH levels of *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice were increased 60- to 160-fold as compared with wild-type mice (Gothe *et al.*, 1999; Furumoto *et al.*, 2005). Serum total T4 and T3 concentrations are increased 18- to 26-fold, respectively, in adult *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice as compared with WT-mice (Furumoto *et al.*, 2005). Consistent with the elevated TSH levels, TSH signaling was increased as indicated by increased phosphorylation of CREB at ages 5–6 and 11–13 months (two- to threefold; Figure 5Aa). Total CREB protein levels were not significantly affected in mice with same age (Figure 5Ab). The key cell-cycle regulator, cyclin D1, which is a downstream effector of TSH–TSH receptor- phosphorylation of CREB signaling was also increased in the protein level (two- to threefold; Figure 5Ba).

To examine whether increased cyclin D1 led to increased cell-cycle progression, we examined whether the protein level of cyclin-dependent kinase 4 was altered. Indeed, as shown in Figure 5Bb, the protein abundance of cyclin-dependent kinase 4 was increased 1.8- to 3-fold in the thyroids of *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice (compare lanes 3 and 4 with lanes 1 and 2; 7 and 8 with 5 and 6). Importantly, increased cyclin D1 and cyclin-dependent kinase 4 led to an increase in phospphorylated retinoblastoma protein in the thyroids of *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice (p-Rb; lanes 3, 4, 7 and 8, S780 in 5Bc and S807/811 in 5Bd). Increased phosphorylation of Rb leads to release the associated-E2F from unphosphorylated Rb-E2F complexes to drive the expression of transcription factors, thereby propelling cells to enter the S-phase to increase cell proliferation (Herwig and Strauss, 1997).

To elucidate the changes in the pathways that have been reported to contribute to thyroid carcinogenesis, we first examined the PI3K–AKT signaling in *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice. This pathway is critical for tumor proliferation, and its dysregulation in tumors is common (Ringel *et al.*, 2001; Miyakawa *et al.*, 2003). As shown in Figure 6Aa, phosphorylated AKT in *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice was increased 3- to 3.5-fold (lanes 3, 4, 7 and 8) as compared with the WT-mice (lanes 1, 2, 5 and 6) without significantly affecting the total AKT protein levels (Figure 6Ab). Consistent with the activation of AKT, phosphorylated mTOR at S2448 and at S2481 were increased 1.5- to 2.5-fold in *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice as compared with WT-mice (Figure 6Ba and b, respectively). A ~twofold increased phosphorylated p70^{S6K} was observed in thyroids of *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice as compared with WT-mice (Figure 6Bd). No significant changes in the protein abundance of total mTOR (Figure 6Bc) as well as total p70^{S6K} were noted (Figure 6Be). The activation of the AKT–mTOR–p70^{S6K} pathway promotes growth of thyroid tumors.

The tumor suppressor p53 induces apoptosis in response to oncogenic transformation (Sharpless and DePinho, 2002). We, therefore, determined whether p53 protein levels were changed to contribute to the growth of thyroid tumors. A weak basal level of p53 was observed in WT-mice aged 5–6 and 11–13 months (lanes 1, 2, 5 and 6, Figure 6Ca). However, no p53 could be detected in the thyroid of *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice at these two ages (lanes 3, 4, 7 and 8, Figure 6Ca) under the same experimental conditions. The apparent reduced p53 protein level prompted us to examine the expression of its regulator, murine double minute oncogene protein (MDM2). MDM2 acts as a p53 ubiquitin ligase to increase proteasome degradation of p53. Figure 6Cb shows that the MDM2 protein level was markedly elevated eight-to ninefold as compared with that in WT-mice at ages 5–6 and 11–13 months (Figure 6C), indicating that the reduced p53 protein level observed in Figure 6Ca was due at least partially to increase proteasomal degradation of p53. Thus, decreased

apoptosis mediated by the lowered p53 level could also contribute to the aberrant thyroid growth of $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice shown in Figure 2.

We next determined whether the expression of the peroxisome proliferator-activated receptor γ (PPARγ) was altered in the thyroid of $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice during thyroid carcinogenesis. PPARγ has been suggested as a tumor suppressor in human follicular thyroid cancer (Shen and Chung, 2005; Teresi and Waite, 2008). PPARγ was shown to suppress proliferation and increase apoptosis of thyroid tumor cells by activating nuclear factor-κB signaling (Kato *et al.*, 2006). Western blot analyses showed that the protein abundance of PPARγ in the thyroid of ageing $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice was progressively reduced as compared with WT-mice (Figure 6D). Approximately, 40 and 80% reductions of PPARγ protein level were detected at ages 5–6 and 11–13 months, respectively (Figure 6Da). These results indicate that during thyroid carcinogenesis, the expression of PPARγ protein remains low, consistent with the notion that PPARγ could function as a tumor suppressor during thyroid carcinogenesis.

Activation of signaling pathway to increase cell invasion and angiogenesis in thyroids of $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice

To determine the changes in the signaling pathways leading to increase cell invasion and migration, we analyzed the expression of key effectors in ILK–MMP-2 signaling pathway. ILK is a ubiquitously expressed protein serine-threonine kinase that binds to integrins. The kinase activity of ILK is stimulated by integrins or growth factors in a PI3K–AKT-dependent manner (Persad and Dedhar, 2003; Troussard *et al.*, 2003). Increased ILK expression and activity result in invasive and metastatic phenotypes (Troussard *et al.*, 2000). MMP-2 is critically involved in degradation of extra-cellular matrix (Brinckerhoff and Matrisian, 2002; Turpeenniemi-Hujanen, 2005).

The AKT signaling that was activated in the thyroid of $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice prompted us to determine whether ILK–MMP-2 was overexpressed. Figure 7A shows that 1.5- and 3-fold increases in the activation of ILK were detected in $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice at 5–6 and 11–13 months, respectively (Figure 7Aa). The abundance of MMP-2 protein, the direct downstream target of ILK, was increased 1.6- to 2-fold for $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice at 5–6 and 11–13 months, respectively (Figure 7Ab). These results indicate an increased activation of ILK–MMP-2 signaling as carcinogenesis progressed, thus, contributing to invasion and metastasis of thyroid tumor cells in $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice.

Angiogenesis is critical for the growth and metastatic spread of tumors. Vascular endothelial growth factor (VEGF) is the most potent inducer of neovasculature, and its increased expression has been associated with the development of metastases (Weidner *et al.*, 1991) and reduced survival (Maeda *et al.*, 1995). We, therefore, studied the expression of VEGF and its receptor, VEGFR2, in the thyroid of $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice. Figure 7Ba shows that the abundance of VEGF was 4.9- and 5.8-fold higher than in WT-mice at 5–6 and 11–13 months, respectively. A higher expression of VEGF at an advanced stage of carcinogenesis is consistent with an increase in the occurrence frequency of vascular invasion as mice aged (see Figure 4B).

Using antibodies against VEGFR2 protein, we detected two bands with slightly different molecular weights in the thyroid of $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice (Figure 7Bb), indicative of the phosphorylated VEGFR2 with a higher molecular weight and the non-phosphorylated form with a lower molecular weight (lanes 3, 4, 7 and 8). In contrast, only the lower non-phosphorylated form of VEGFR2 was detected in the WT-mice (lanes 1, 2, 5 and 6, Figure 7Bb). That VEGFR2 protein with a higher molecular weight band was the activated phosphorylated form was further confirmed by using antibody specific for the

phosphorylated VEGFR2 protein with which only the higher molecular weight band was detected in *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice, but not in WT-mice (Figure 7Bc). All together, the activated VEGF–VEGFR2 signaling promotes the angiogenesis and invasion of tumor cells.

The pituitary tumor-transformation gene (*PTTG*), originally isolated from GH4 pituitary cells, has been shown to cause cell transformation *in vitro* and to induce tumor formation *in vivo* (Pei and Melmed, 1997). Its overexpression occurs in a wide variety of non-endocrine and endocrine tumors including thyroid cancer (Heaney *et al.*, 2001; Kim *et al.*, 2003). PTTG is involved in multiple cellular pathways, including proliferation, DNA repair, transformation, angiogenesis induction, invasion and the induction of genetic instability. Recently, overexpression of the PTTG protein has been shown to induce cell-cycle abnormalities and aneuploidy in thyroid follicular cells (Ying *et al.*, 2003a, 2006; Zimonjic *et al.*, 2005) and to promote angiogenesis in thyroid tumor cells (Kim *et al.*, 2007). In thyroid carcinomas, PTTG expression is a marker of invasiveness. We, therefore, examined the protein abundance of PTTG in the thyroid of *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice during thyroid carcinogenesis. Figure 7C shows that, similar to human thyroid cancer, PTTG protein abundance was elevated approximately twofold in the thyroid tumors of *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice at ages 5–6 months (lanes 3 and 4) and 11–13 months (lanes 7 and 8) as compared with WT-mice at the corresponding ages (lanes 1 and 2, and 5 and 6). These results suggest that overexpressed PTTG could increase angiogenesis and invasion to promote tumor progression.

Discussion

This study shows that *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice spontaneously developed metastatic FTC as they aged. Extensive molecular analyses showed that complex alterations of multiple signaling pathways contribute to the development and progression of metastatic carcinoma (see Figure 8). Thyroid growth was stimulated by multiple proliferation signals, such as the TSH–TSHR–pCREB–cyclin D1 pathway and AKT–mTOR–p70^{S6K} signaling. Several pathways known to promote cell invasion and motility, such as ILK–MMP-2 and PTTG, were also activated in the thyroid of *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice. Consistent with reports by others (McCabe *et al.*, 2002; Kim *et al.*, 2006), we also found that an elevated PTTG was accompanied by induction of VEGF and VEGFR, thereby increasing the angiogenesis of tumor cells to further promote tumor progression (Figure 8). The decreased p53 is known to decrease apoptosis. The end result of these multiple alterations is development and progression of thyroid cancer.

Currently, the molecular details by which the loss of both functional TRs mediates the changes of the key effectors affecting the cellular signaling in growth, invasion and angiogenesis remain to be fully elucidated. However, TRs are known to act (directly and indirectly) through genomic and nongenomic actions to regulate target genes expression and activities. Thus, through genomic actions, the loss of TRs increases the expression of negatively regulated genes. Examples are the increased expression of *TSHα* and *TSHβ* genes (TR direct target genes) in the pituitary to stimulate thyroid growth and the increased expression of cyclin D1 in the thyroid (through indirect regulation) to increase tumor cell proliferation through cyclin-CDK4–pRb signaling (Furumoto *et al.*, 2005). However, understanding of how the loss of TRs results in altered expression and activities of other key regulators and in the activation of phosphorylation cascades (for example, AKT–mTOR–p70^{S6K}) awaits further studies. Importantly, however, the phenotypic alterations resulting in carcinogenesis because of the loss of normal functions of TRs are consistent with the hypothesis that TRs could function as tumor suppressors in the development of thyroid cancer in *TRα1*$^{-/-}$*TRβ*$^{-/-}$ mice.

TSH has long been known as a major stimulator of thyrocyte proliferation. Whether it is an initiator of thyroid carcinogenesis, however, remains to be clarified. Approximately, 30% of rats fed an iodine-deficient diet developed follicular carcinomas by 18 months, but no metastatic tumors were found (Ward and Ohshima, 1986). Several studies also showed a more prevalent occurrence of follicular carcinomas in patients in iodine-deficient regions (Lawal *et al.*, 2001). These findings suggest the possibility that TSH could initiate thyroid carcinogenesis, but recent studies provide compelling evidence to the contrary. Transgenic mice with thyroid-specific expression of the A2 adenosine receptor (Ledent *et al.*, 1992), a mutated $G_s\alpha$ (Michiels *et al.*, 1994) or cholera toxin A1 (Zeiger *et al.*, 1997) develop thyroid hyperplasia and hyperthyroidism, but not carcinomas. In addition, patients with Graves' disease or with congenital hyperthyroidism because of germline mutations of the TSH receptor do not appear to have a higher rate of thyroid malignancy compared with persons with normal TSH levels (Fagin, 2002). These studies suggest that growth signals provided by TSH are necessary for thyrocyte proliferation, but not sufficient for metastatic carcinoma to occur. Additional genetic changes would need to occur for the transformation of the hyperproliferative thyroid cells to cancer cells. In $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice, the loss of both functional TR isoforms results in total loss of negative regulation of the pituitary-thyroid axis, thereby elevating and sustaining high serum TSH levels to provide proliferation signals to the follicular cells (60- to 160-fold higher than in WT-mice; (Gothe *et al.*, 1999; Furumoto *et al.*, 2005)). In addition, the regulatory activities of TRs necessary to maintain normal cellular functions are lost, leading to disarrayed signaling to develop metastatic tumors. Indeed, $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice showed extensive hyperplasia as early as 2–3 months of age, preceding the occurrence of capsular and vascular invasion and distant metastasis at a later stage of cancer progression (see Figure 4). These observations suggest that growth and metastasis are two related, but independent processes. The former is a prerequisite for the latter to occur. However, without additional genetic alterations to enable the latter to occur, there would be no metastatic carcinoma.

This molecular model of thyroid carcinogenesis is supported by the phenotypic expression of several genetically engineered mice. So far, no thyroid cancer has been reported for mice deficient in *TR*β ($TR\beta^{-/-}$ mice) or *TR*α*1* ($TR\alpha 1^{-/-}$ mice) alone. The $TR\beta^{-/-}$ mice show mild resistance to thyroid hormone with elevation of two- to threefold of thyroid hormones accompanied by a moderately increased TSH (two- to threefold as compared with WT-mice; (Forrest *et al.*, 1996b; Forrest and Vennstrom, 2000). Mice lacking TRα1 show a mild hypothyroidism with nearly normal TSH levels (Wikstrom *et al.*, 1998). Therefore, in these two mutant mice, there is no strong TSH proliferation signal to sustain long-term aberrant growth in spite of the loss of the suppressor functions of either TRβ or TRα1. $TR\beta^{PV/PV}$ mice (Suzuki *et al.*, 2002) and $TR\beta^{PV/-}$ mice (a mutated *TR*β gene together with a loss of a wild-type *TR*β gene) are known to spontaneously develop metastatic FTC (Kato *et al.*, 2004). $TR\beta^{PV/PV}$ and $TR\beta^{PV/-}$ mice show similarly highly elevated TSH levels to drive the growth of follicular cells (Suzuki *et al.*, 2002; Kato *et al.*, 2004). The loss of the suppressor functions of TRβ because of mutations of the two *TR*β alleles, as in $TR\beta^{PV/PV}$ mice, or the mutation of one allele of the *TR*β gene together with the loss of the other allele, as in $TR\beta^{PV/-}$ mice, empowers the hyperplastic follicular cells to progress to metastatic carcinoma.

The deleterious effects of mutations or the loss of the *TR*β gene in the thyroid is evident in the $TR\beta^{PV/PV}$ mice (Suzuki *et al.*, 2002) and $TR\beta^{PV/-}$ mice (Kato *et al.*, 2004). Less clear are the consequences of loss of TRα1 in the thyroid during carcinogenesis. It is known that the major TR isoform in the thyroid is TRβ (Ying *et al.*, 2003b). Therefore, it is possible that in $TR\beta^{PV/PV}$ mice and $TR\beta^{PV/-}$ mice, normal functions of TRα1 are also inhibited by TRβPV through the dominant negative effect as similarly as shown in the liver (Zhang *et al.*, 2002). Thus, the normal activities of TRα1 are lost in the thyroids of $TR\beta^{PV/PV}$ and

$TR\beta^{PV/-}$ mice as in those of $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice, although the underlying mechanisms are different. In $TR\beta^{PV/PV}$ and $TR\beta^{PV/-}$ mice, the activities of TRα1 are inactivated because of the potent dominant negative activity of TRβPV, whereas in $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice, it is the deletion of both of the alleles of the *TR*α gene. These results would indicate that both TRs are critically important to maintain normal functions of the thyroid. Their loss by mutations of the *TR*β gene or deletion of the *TR*β and the *TR*α genes would propel hyperplastic thyroid cells to become metastatic cancer cells. Thus, this study has revealed a novel tumor suppressor role of TRs in thyroid carcinogenesis.

Materials and methods

Experimental animals

Animal experiments were performed according to the protocols approved by the Animal Care and Use Committee at the National Cancer Institute. Mice deficient in *TR*α*1* and *TR*β genes were genotyped as described (Forrest *et al.*, 1996a; Wikstrom *et al.*, 1998). Heterozygous *TR*α*1*- and *TR*β- deficient mice were intercrossed to generate WT- and $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice. Thyroids and other tissues were harvested from $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice and WT-mice littermates for weighing, histological analyses and biochemical studies.

Western blot analysis

Thyroids dissected from $TR\alpha 1^{-/-}TR\beta^{-/-}$ mice and wild-type siblings were washed with phosphate-buffered saline and homogenized in a solution with 50 mM Tris buffer, 150 mM NaCl, 1 mM ethylene-diaminetetraacetic acid, 1% NP40 and proteinase/phosphatase inhibitors. The western blot analysis was carried out as described by Furumoto *et al.* (Furumoto *et al.*, 2005). Primary antibodies for phosphorylated-S473 AKT (#9271), total AKT (#9272), phosphorylated-T421/S424 $p70^{S6K}$ (#9204), total $p70^{S6K}$ (#9202), CREB (#9197), phospho-CREB (#9198), phospho-mTOR (Ser2448) (#2971), phospho-mTOR (Ser2481) (#2974), cyclin-dependent kinase-4 (DCS156) (#2906), retinoblastoma protein (Rb:S807/811, #9307S; S780, #9308S), phospho-VEGFR2 (Tyr1175) (#2478S) and GAPDH (#2118) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-MMP2 (SC-10736), MDM2 (sc-965), VEGF (sc-507), VEGFR2(C17) (sc-316), PPARγ (sc-7196), p53 (sc-6243), Cyclin D1 (sc-450) and ILK (sc-13075) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-PTTG-1 (34–1500) was purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). Anti-mTOR antibody (T2949) was purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies were used at a concentration recommended by manufacturers. For control of protein loading, the blots were probed with the antibodies against GAPDH.

Histological analysis

Thyroids and lungs were dissected and embedded in paraffin. Five-micrometer-thick sections were prepared and stained with hematoxylin and eosin. For each animal, single random sections through the thyroid (usually both lobes), lung and heart were examined. For thyroids, single section hyperplasia, capsular invasion, vascular invasion and anaplasia were routinely evaluated and scored. Hyperplasia was generally diffuse throughout the gland. Evidence of any of these changes in any section was counted as positive for that change. On average, in those cases with capsular invasion and/or vascular invasion, these morphological changes were observed in multiple locations (usually two or three) in any one single thyroid section. The presence of a single microscopic focus of metastatic follicular carcinoma in the lung was counted as positive for metastasis in that animal.

Statistical analysis

Data are expressed as means ± standard errors. Statistical analysis was performed with the use of analysis of variance, and $P < 0.05$ was considered significant unless otherwise specified. StatView 5.0 (SAS Institute Inc., Cary, NC, USA) was used to perform Kaplan-Meier cumulative survival analysis, and Student's *t*-test using odds ratios and Fisher's exact probability test were used to analyze the data of pathological progression. PRISM 4.0a (GraphPad Software, San Diego, CA, USA) was used for log-rank testing for statistical significance.

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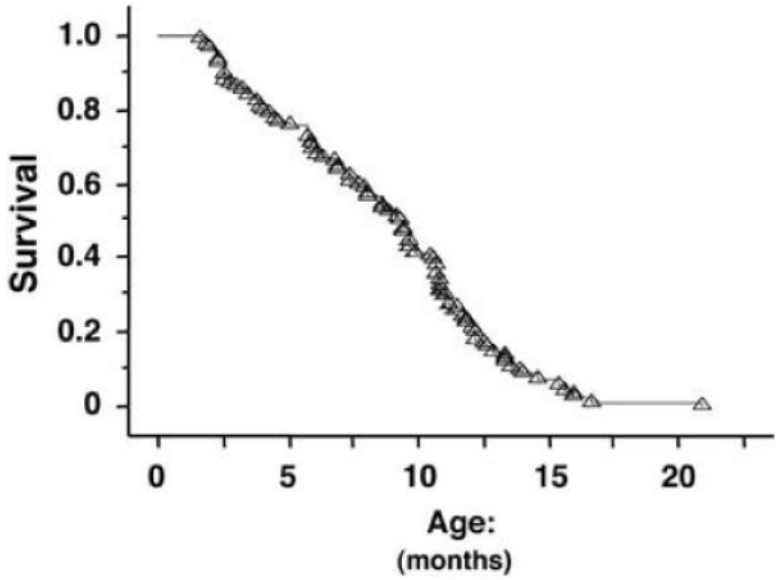

Figure 1.
Kaplan–Meier survival curve for *TRα1*$^{-/-}$ *TRβ*$^{-/-}$ mice up to 20.9 months of age. The analysis was performed with log-rank (Mantel–Cox) test by using StatView 5.0. The 50% survival age was 9.3 months (n=94).

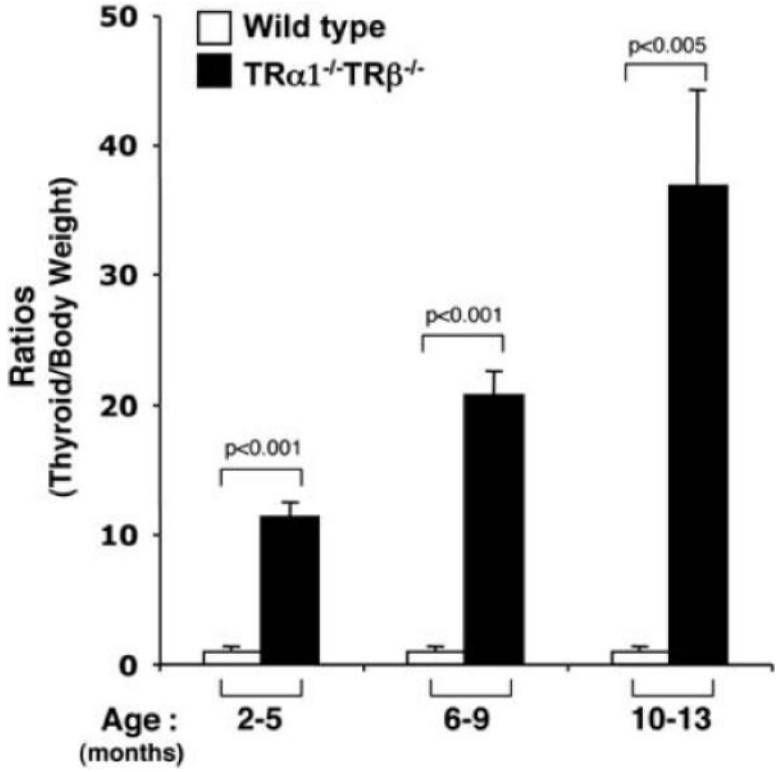

Figure 2.
Thyroid weights of *TRα1*$^{-/-}$ *TR*β$^{-/-}$ mice (*n*=6–25) at the ages of 2–5, 6–9 and 10–13 months. Thyroid glands of *TRα1*$^{-/-}$ *TR*β$^{-/-}$ mice were dissected and weighed. The data are presented as the ratios of thyroid weight to body weight (mg/g).

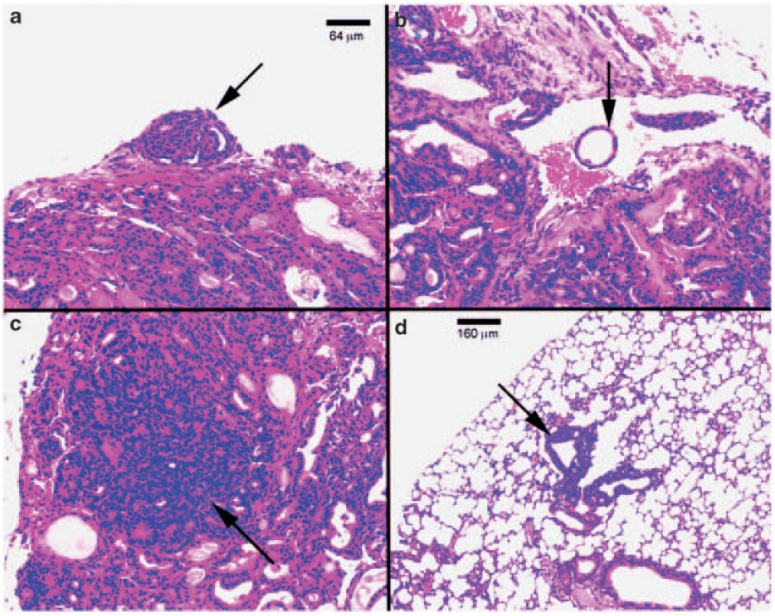

Figure 3.

Hematoxylin and eosin (H&E) staining of thyroids and lungs of representative sections from $TR\alpha 1^{-/-}$ $TR\beta^{-/-}$ mice. Histological sections from tissues of mice showed evidence of capsular invasion in thyroid (**a**) (arrow), vascular invasion in thyroid (**b**) (arrow), anaplasia in thyroid (**c**) and metastatic thyroid carcinoma lesions in lung (**d**) (arrow).

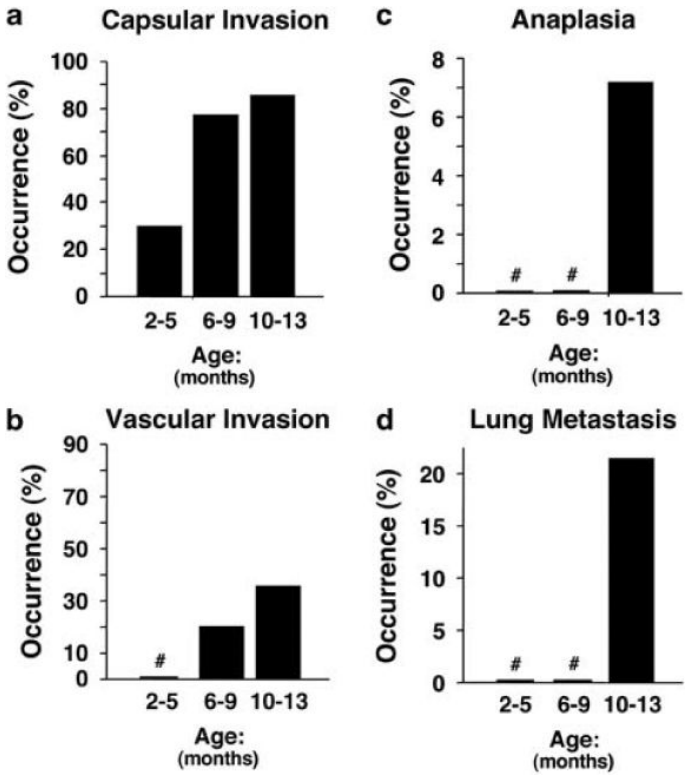

Figure 4.

Quantitative analysis of age-dependent occurrence frequency (%) of capsular invasion (**a**), vascular invasion (**b**), anaplasia (**c**) and lung metastasis (**d**) of *TRα1*$^{-/-}$ *TRβ*$^{-/-}$ (n=6–14) mice. Sections of thyroids and lungs from *TRα1*$^{-/-}$ *TRβ*$^{-/-}$ mice were stained with Hematoxylin and eosin (H&E) and analyzed for age-dependent pathological progression. The data are expressed as the percentage of occurrence frequency of the mice examined. The designation (#) indicates 0 occurrence frequency (%).

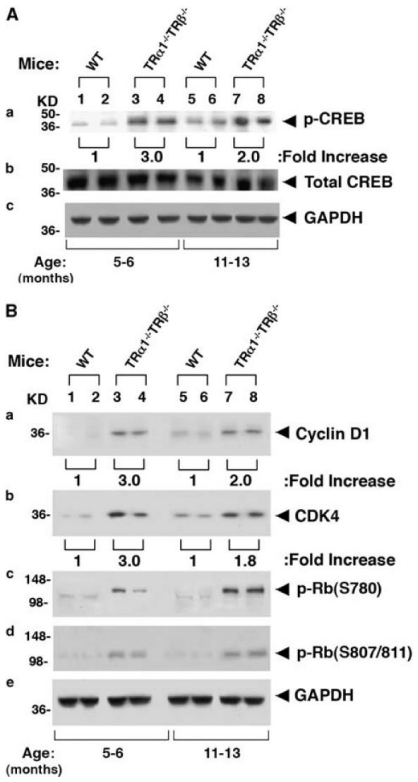

Figure 5.

Activation of TSH–TSHR downstream pathway (**A**) and cyclin-CDK4–Rb pathway (**B**) in *TRα1*$^{-/-}$ *TRβ*$^{-/-}$ mice. For western blot analysis, 30 μg of thyroid extract was used. Two representative results from 4–6 WT (lanes 1, 2, 5 and 6) and *TRα1*$^{-/-}$ *TRβ*$^{-/-}$ mice (lanes 3, 4, 7 and 8) are shown for p-CREB and total CREB (**A**), cyclin D1, CDK4 and p-Rb (**B**). GAPDH was used as loading controls.

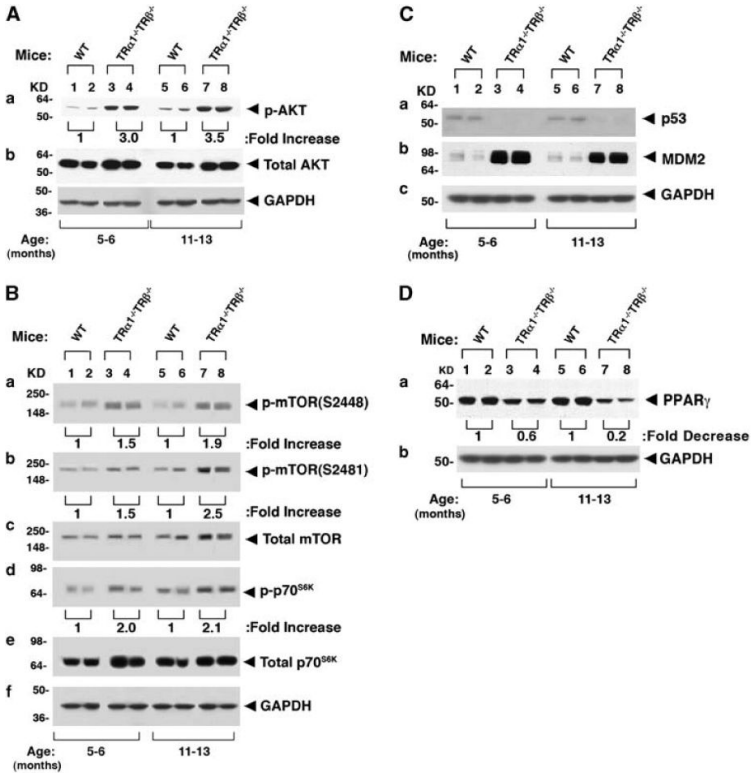

Figure 6.

Activation of growth signaling pathways in *TRα1*$^{-/-}$ *TRβ*$^{-/-}$ mice. For western blot analysis, 30 μg of thyroid extract was used. Two representative results from 4 to 6 WT (lanes 1, 2, 5 and 6) and *TRα1*$^{-/-}$ *TRβ*$^{-/-}$ mice (lanes 3, 4, 7 and 8) are shown for p-AKT and total AKT (**A**), p-mTOR(S2448), p-mTOR(S2481), total mTOR, p-p70^{S6K} and total 70^{S6K} (**B**), p53 and MDM2 (**C**) and PPARγ (**D**). GAPDH was used as loading controls.

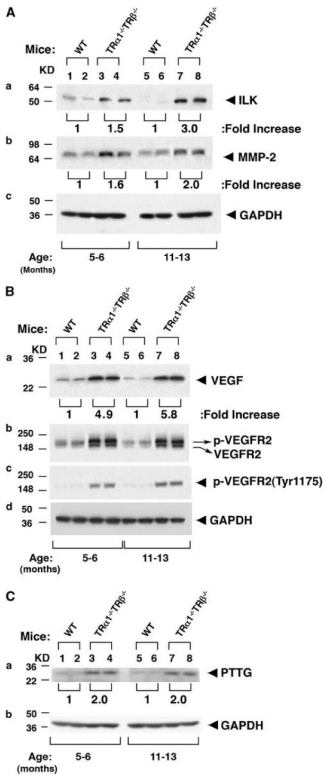

Figure 7.

Activation of pathways involved in invasion and angiogenesis. Thyroid extract (30 μg) was used in the western blot analysis, as described in Materials and methods section. Two representative results from 5 to 7 WT (lanes 1, 2, 5 and 6), *TRα1*$^{-/-}$ *TRβ*$^{-/-}$ mice (lanes 3, 4, 7 and 8) are shown for ILK and MMP-2 (**A**), VEGF, VEGFR2 and p-VEGFR2 (*B*), and PTTG (**C**). GAPDH was used as loading controls.

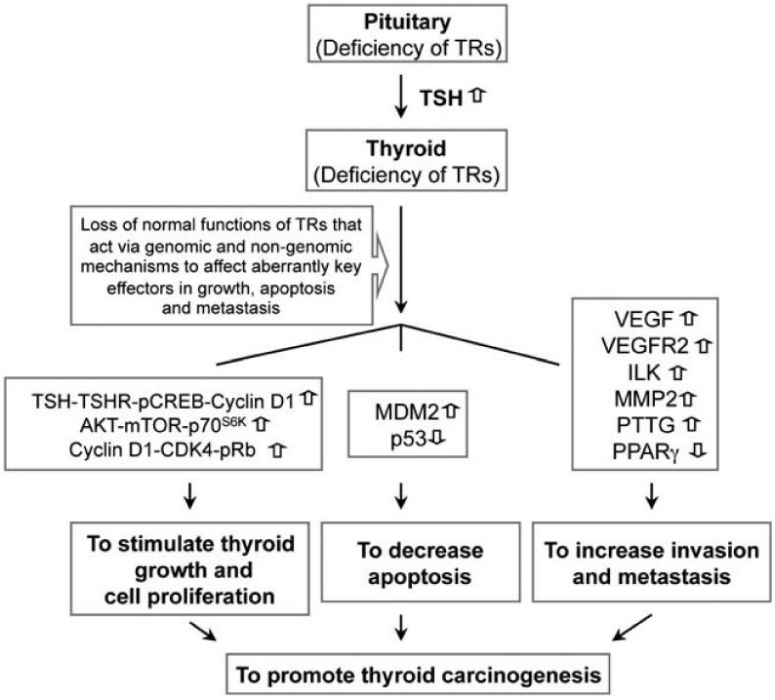

Figure 8.

The loss of normal TR functions results in complex alterations of multiple signaling pathways, thereby contributing to thyroid carcinogenesis in $TR\alpha 1^{-/-}$ $TR\beta^{-/-}$ mice. TRs act through genomic and nongenomic actions to regulate key cellular effectors to maintain normal cellular functions in growth, proliferation, cell motility and migration. TRs through T3 repress the expression of TSHα and TSHβ genes in the pituitary. Deficiency of TRs in the pituitary results in the loss of negative regulation and thus increases expression of TSH. Although little is known regarding the precise mechanisms by which TRs regulate other key effectors, such as VEGFR2 and others indicated in this figure (see also Discussion section), the loss of functional TRs results in disarrays of multiple signaling pathways to contribute to thyroid carcinogenesis. The phenotypic manifestation of tumor development and progression because of the loss of normal functions of TR is consistent with the notion that TRs have key tumor suppressor roles.