Oncogenic Actions of the Nuclear Receptor Corepressor (NCOR1) in a Mouse Model of Thyroid Cancer

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Introduction

Thyroid hormone receptors (TRs) are critical in mediating the genomic actions of the thyroid hormone (T3) in growth, development, differentiation, and maintaining metabolic homeostasis. Two TR genes, α and β, located on two different chromosomes, encode three major T3 binding TR isoforms (α1, β1, and β2). Studies using genetically engineered mice showed that, in vivo, TR isoforms have common functions, but can also exert isoform-dependent actions in target tissues [1,2]. The transcription activity of TR is regulated at multiple levels. In addition to the regulation by T3 and types of DNA binding elements in the promoters of target genes, the transcriptional activity of TR is fine-tuned by a host of nuclear coactivators and corepressors [3,4]. In the absence of T3, the unliganded TR recruits the nuclear receptor corepressor 1 (NCOR1) and the nuclear receptor corepressor 2/silencing mediator for retinoid and thyroid hormone receptors (NCOR2/SMRT) for transcriptional repression. Binding of T3 leads to a conformational change in the TR that releases the NCOR1/NCOR2 complex and allows for the recruitment of a multiprotein coactivator complex for transcriptional activation [5].

The important regulatory role of NCOR1 in receptor actions is evident in its aberrant interaction with receptors underlying human diseases such as resistance to thyroid hormone (RTH) [6,7] and lipodystrophic severe insulin resistance [8]. RTH is caused by mutations in the THRB gene [9,10]. Mutations of the peroxisome proliferator-activated receptor γ (PPARγ) lead to lipodystrophic severe insulin resistance [8]. In addition, aberrant interaction of NCOR1/SMRT with the fused gene products of the retinoic acid receptor α gene (RARα) or with the promyelocytic leukemia gene (PML) (PML-RAR-α) or with the promyelocytic leukemia zinc finger gene (PLZF) (PLZF-RAR-α) results in blocking myeloid differentiation [11]. The involvement of NCOR1 in other human cancers such as breast and bladder cancers was demonstrated by a close association of NCOR1 abnormal expression with cancer development [12,13,14,15]. Recent studies by The Cancer Genome Atlas (TCGA) Research Network also uncovered homozygous gene deletion of NCOR1 in some patients with colon and rectum adenocarcinoma [16], prostate adenocarcinoma [17], ovarian carcinoma [18] or liver hepatocellular carcinoma (unpublished-Provisional in TCGA data base). Moreover, nonsense mutations and splicing variants of NCOR1 were also found in patients with breast cancer [19]. While these association studies raised the possibility that NCOR1 could act to affect cancer progression, direct evidence of its roles in carcinogenesis in vivo is still lacking.

The availability of a mouse model that spontaneously develops metastatic follicular thyroid cancer (FTC) provided us with a

Abstract

Studies have suggested that the nuclear receptor corepressor 1 (NCOR1) could play an important role in human cancers. However, the detailed molecular mechanisms by which it functions in vivo to affect cancer progression are not clear. The present study elucidated the in vivo actions of NCOR1 in carcinogenesis using a mouse model (ThrbPV/PV mice) that spontaneously develops thyroid cancer. ThrbPV/PV mice harbor a dominantly negative thyroid hormone receptor β (TRβ) mutant (denoted as PV). We adopted the loss-of-the-function approach by crossing ThrbPV mice with mice that globally express an NCOR1 mutant protein (NCOR1ΔID) in which the receptor interaction domains have been modified so that it cannot interact with the TRβ, or PV, in mice. Remarkably, expression of NCOR1ΔID protein reduced thyroid tumor growth, markedly delayed tumor progression, and prolonged survival of ThrbPV/PVNCOR1ΔID mice. Tumor cell proliferation was inhibited by increased expression of cyclin-dependent kinase inhibitor 1 (p21<sup>Waf1/Cip1</sup>), Cdkn1A, and apoptosis was activated by elevated expression of pro-apoptotic BCL-Associated X (Bax). Further analyses showed that p53 was recruited to the p53-binding site on the proximal promoter of the Cdkn1A and the Bax gene as a co-repressor complex with PV/NCOR1/histone deacetylase-3 (HDAC-3), leading to repression of the Cdkn1A as well as the Bax gene in thyroids of ThrbPV/PV mice. In thyroids of ThrbPV/PVNCOR1ΔID mice, the p53/PV complex could not recruit NCOR1ΔID and HDAC-3, leading to de-repression of both genes to inhibit cancer progression. The present studies provided direct evidence in vivo that NCOR1 could function as an oncogene via transcription regulation in a mouse model of thyroid cancer.

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powerful tool to assess the role of NCOR1 in cancer development and progression. The ThrbPV/PV mouse harbors a knockin dominant negative mutation, known as PV, in the Thrb gene locus [20]. The PV mutation was identified in a patient suffering from resistance to thyroid hormone [21]. As ThrbPV/PV mice age, their thyroids undergo pathological changes from hyperplasia to capsular and vascular invasion, anaplasia, and eventual metastasis to the lung [22]. The pathological progression, route, and frequency of metastasis in ThrbPV/PV mice are similar to that in human FTC. Extensive molecular analyses of altered signaling pathways show that, as found in human FTC, ThrbPV/PV mice exhibit aberrant signaling pathways that include constitutive activation of phosphatidylinositol 3-kinase (PI3K/Akt) [23,24] and integrin–Src–MAPK signaling [25] and aberrant accumulation of the oncogenic pituitary tumor transforming gene protein (PTTG) [26,27] and ß-catenin [28]. Thus, the ThrbPV/PV mouse model faithfully recapitulates the molecular aberrations found in human thyroid cancer and is indeed a preclinical mouse model of FTC.

In the present studies, we adopted the loss-of-the-function approach by crossing ThrbPV/PV with mice that globally express an NCOR1 mutant protein (NCOR1ID). In this NCOR1ID mutant protein, two most amino terminal receptor interaction domains termed RID 3 and RID2, are missing. This mutant cannot associate with TR, as RID3 is absolutely required for NCOR1-TR interaction [29,30,31]. Consistently, we also showed that NCOR1ID mutant protein cannot interact with TRPV in vivo [32]. The lack of interaction of TRβPV with NCOR1ID results in the amelioration of symptoms of resistance to thyroid hormone in ThrbPV/NCOR1ID mice, indicative that NCOR1 regulates the dominant negative action of TRβ mutants in vivo [32].

The present studies show that the expression of NCOR1ID in the thyroid of ThrbPV/PV mice inhibited tumor growth, prolonged survival, and delayed cancer progression. Tumor cell proliferation was inhibited by the increased expression of cyclin-dependent kinase inhibitor 1 (p21/WAF1; Cldn1A), and the apoptosis was induced by the activated expression of the Bcl-2–associated X protein (BAX). Both the Cldn1A and the BAX genes are direct downstream target genes of the tumor suppressor, p53. Detailed molecular analyses showed that the lack of receptor interaction domain in NCOR1ID led to an inability of the p53/PI3K complex to recruit the NCOR1/histone deacetylase-3 repressor complex to the promoters of these target genes, resulting in increased repression of these genes to inhibit cancer progression. The present study provided direct evidence in vivo to demonstrate that the NCOR1 could function as an oncogene via transcription regulation in thyroid carcinogenesis in a mouse model.

Materials and Methods

Mouse Strains

The animal study was carried out according to the protocol approved by the National Cancer Institute Animal Care and Use Committee. Mice harboring the ThrbPV gene (ThrbPV/+ mice) were prepared and genotyped as described earlier [20]. ThrbPV/NCOR1ID mice were bred by first crossing ThrbPV/+NCOR1ID/+ mice [29] with ThrbPV/+ mice and then by crossing ThrbPV/NCOR1ID/+ mice with ThrbPV/+NCOR1ID/+ mice [32]. Mice with different genotypes used in the present study were intercrossed several generations, and littermates with a similar genetic background were used in all experiments. Mice were monitored until they became moribund and therefore euthanized. Thyroids and other tissues were collected from ThrbPV/NCOR1ID mice and ThrbPV/NCOR1ID/ID mice for weighing, histological analysis, and molecular and biochemical studies.

RNA Isolation and Quantitative Real-Time RT-PCR

Total RNA was extracted from thyroids of mice using TRızol (Invitrogen) according to the manufacturer’s instructions. Quantitative real-time RT-PCR was performed with a QIaQuant SYBR Green RT-PCR kit (Qiagen, Valencia, CA); according to the manufacturer’s instructions and using a LightCycler thermal cycler (Roche Diagnostics). Total RNA (200 ng) was used in RT-PCR determinations as described previously [33]. The specific primers were as follows: mp2 forward, 5'-CGGCCGGTGTGCAAGTC-3' and reverse, 5'-GGCGAGGCGAGAAGAACG-3'; mBax forward, 5'-CCACCAAGCTCTGAAAGATC-3' and reverse, 5'-CAGCTTCTTTGTTGTGACGACAT-3'; mp53 forward, 5'-AGAGACGGCGGTAGAGCGAAG-3' and reverse, 5'-CTGTAGCAATGGGCATCCCTT-3'.

Western Blot Analysis and Co-immunoprecipitation

Nuclear extracts of thyroids of mice were prepared as previously described [32]. The protein samples (25 μg) were analyzed by Western blot as described previously [28]. Anti-phosphorylated retinoblastoma protein (pRb) (Ser780, #9307) and PUMA (7467) were from Cell Signaling Technologies (Beverly, MA) (1:300 dilution), anti-cyclin D1 (sc-450), BAX (sc-7467), Rh (sc-50), p21 (sc-6246) and p27 (sc-1641) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used at a 1:200 dilution. Anti-p53 antibodies (OP03) were from Calbiochem (1:500 dilution). Band intensities were quantified by using NIH IMAGE software (Imagej 1.34 s; Wayne Rasband, NIH).

For co-immunoprecipitation to show the physical interaction of p53 with PV, 0.5 mg of total thyroid extracts was first incubated overnight with rabbit anti-TR (Code 600-401-A96, Rockland) in Tris-buffered saline-0.6% NP-40 with protease inhibitors (Roche) at 4°C. The samples were then mixed with 20 μl protein A-agarose (Roche) at 4°C for 2 hours, and beads were washed five times with TBS-0.6% NP-40 containing protease inhibitors. Bound proteins were analyzed by Western blot analysis using antibodies for p53 (OP03; Millipore, Inc.,) at a 1:500 dilution and anti-NCOR1 (PHQ0; 2 μg/ml) [32].

Histological Analysis

Thyroid gland, lung, and heart were dissected, fixed in 10% neutral buffered formalin (Sigma-Aldrich, St. Louis, MO), and subsequently embedded in paraffin. Sections of 5-μm thickness were prepared and stained with hematoxylin and eosin (H&E). For each animal, single random sections through the thyroid, through the lung, and through the heart were examined.

Immunohistochemistry

IHC was performed as previously described with some modification [25]. Formalin-fixed paraffin thyroid sections were deparaffinized, rehydrated, heated to 98°C in 0.05% citric acid anhydride, pH 7.4 (Sigma-Aldrich, St. Louis, MO), for 1 hour and then blocked for 1 hour in 2% normal goat serum at room temperature. After washing in phosphate-buffered saline, slides were incubated at 4°C overnight with Ki-67 primary antibody (1:300 dilution; Thermo Scientific, Fremont, CA; #RB-9043-P0). After washing, slides were incubated with goat anti-rabbit secondary antibody for 1 hour at room temperature and rinsed in phosphate-buffered saline. Slides were then incubated in 3,3'-diaminobenzidine (DAB substrate kit for peroxidase; Vector Laboratories, Burlingame, CA; SK-4100), and after staining...
Role of NCOR1 in Thyroid Carcinogenesis

Chromatin Immunoprecipitation (ChIP) Assay

The ChIP assay was carried out using chromatin DNA prepared from thyroid tumors (50 mg/assay) of ThrbPV/PV\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice and ThrbPV/PV\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice, as described previously [34]. The immunoprecipitated-DNA in 50 μl of TE and quantitative PCR was performed with 7900HT Fast Real-Time PCR System (Applied Biosystems) using QuantiFast SYBR Green RT-PCR Kit (204154, QIAGEN). The enrichment in signals was calculated as immunoprecipitation signals versus whole cell lysate inputs. The fold changes of thyroid tumors of ThrbPV/PV\textsuperscript{NCOR1\textsuperscript{AD/AD}} or ThrbPV/PV\textsuperscript{NCOR1\textsuperscript{AD/ID}} ChIP were normalized by negative control (mouse IgG). The ChIP primers used were Cds1A forward, 5’- TTCTTATCGCCGCCAGGA-3’; and reverse, 5’- TACCCCAACAGCTGGTAGTT-3’ and Bax forward, 5’- GGGGCCGCCGCGTACATTCC-3’; and reverse, 5’- GCTTCGTAGGACAGGGGC-3’.

Statistical Analysis

All data were expressed as mean ± SEM. Differences between groups were examined for statistical significance using Student’s t-test with the use of GraphPad Prism 4.0a (GraphPad Software); p<0.05 is considered statistically significant.

Results

NCOR1\textsuperscript{AD} Prolongs Survival and Delays Thyroid Carcinogenesis of Thrb\textsuperscript{PV/PV} Mice

We have previously shown that Thrb\textsuperscript{PV/PV} mice have high mortality caused by FTC [22]. We monitored the effects of the expression of NCOR1\textsuperscript{AD} on thyroid carcinogenesis by first comparing the survival of Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice with that of Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice. Mice were monitored until they became moribund with signs of palpable tumors, rapid weight loss, hunched postures, and labored breathing. Figure 1A shows that Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice survived significantly longer (p<0.01; 50% survival age: 11.3 months, n = 29) than did Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice (50% survival age: 9.3 months, n = 58) during the 15-month observation period. Figure 1B shows that the expression of NCOR1\textsuperscript{AD} led to a significant 35% reduction in thyroid weight in Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice (data set 2 vs. 1; p<0.0001).

The effect of NCOR1\textsuperscript{AD} on the pathological progression was assessed by histopathological analysis as the Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice aged. Figure 2A shows representative pathological features of the thyroids and lumps from the 3–13-month-old Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} and Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice (left and right columns, respectively). In the thyroids of Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice at the age of 7 months, advanced vascular invasion and focal anaplasia were frequently observed (arrows in Figures 2Aa and 2Ac, respectively). Moreover, lung metastases (Figure 2Ae) were frequent in Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice. In Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice, however, vascular invasion was rarely observed (Figure 2Ab) and a marked reduction in the occurrence of lung metastasis (Figure 2Ad). These pathological observations are summarized in Figure 2B. At the younger age of 3–5 months, a lower occurrence of capsular invasion (~20%) was observed for Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice than for Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice (Figure 2Ba), but a similar occurrence frequency of capsular invasion was detected for both mutant mice at older age (>7 months of age). No vascular invasion, anaplasia, or lung metastases were found in Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice (Figure 2Bb, c, and d) at younger ages of 3–5 months. For mice older than 7 months, the occurrence frequency of vascular invasion, anaplasia, and lung metastasis were 80%, 15%, and 60%, respectively, for Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice (Figure 2Bb, c, and d). However, the occurrence of vascular invasion and lung metastasis were 14% and 10%, respectively, for Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice, with no occurrence of anaplasia. Taken together, these results indicate that the expression of NCOR1\textsuperscript{AD} delayed thyroid cancer progression and blocked loss of differentiation (anaplasia) in Thrb\textsuperscript{PV/PV} mice.

NCOR1\textsuperscript{AD} Reduces Thyroid Growth by Inhibiting Cell Proliferation in Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} Mice

The fact that thyroid growth was reduced in Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice (Figure 1B) prompted us to ask whether thyroid cell proliferation was inhibited. We therefore examined the protein abundance of the nuclear proliferation marker, Ki-67, by immunohistochemical analysis. Figure 3A-I-a and -b show representative examples of intensive nuclear staining in thyroids of two Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice. In contrast, fewer nuclei were immunostained with Ki-67 proliferation marker in thyroids of Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice (Figure 3A-I-c and -d, n = 2). Cells with Ki-67 immuno-stained nuclei were counted and the quantitative data are shown in Figure 3A-II. The quantitative analysis shows that the number of thyroid cells with Ki-67 stained nuclei was 50% lower in Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice than in Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice, indicating decreased cell proliferation in the thyroid of Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice.

This finding was further supported by the biochemical analysis in which the protein abundance of a key cell cycle regulator, cyclin D1, was markedly lower in the thyroids of Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice (Figure 3B-I, panel a, lanes 3 & 4) than in Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice (lanes 1 & 2). Moreover, the reduced cyclin D1 led to a lower protein abundance of phosphorylated retinoblastoma protein (pRb) (Figure 3B-I, panel b, compare lanes 1 & 2 with lanes 3 & 4) without changing the total Rb protein levels (panel c). The reduction in the phosphorylated Rb impeded the progression of the cell cycle from the G1 to the S phase. Consistently, the protein abundance of cyclin-dependent kinase inhibitor p21 and p27 (Figure 3B-I, panels d and e, respectively) was also lower in the thyroids of Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice than in Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice (compare lanes 3–4 to lanes 1–2). The quantitative data of the band intensities are shown in Figure 3B-II. Collectively, these results indicate that the expression of the NCOR1\textsuperscript{AD} led to inhibition of tumor cell proliferation, in part, by delaying the G1-S cell cycle progression.

Whether apoptosis also contributed to decreased thyroid tumor growth shown in Figure 1B was also evaluated by examining the key regulators of apoptosis. The protein abundance of Bcl-2–associated X protein (BAX), which promotes apoptosis, was higher in thyroids of Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice than in Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice (Figure 4Aa, compare lanes 3 & 4 with 1 & 2). PUMA, which is a BH3 (Bcl-2 homology domain 3)-only protein that induces apoptosis through the mitochondria pathway, was also higher in thyroids of Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/AD}} mice than in Thrb\textsuperscript{PV/PV}\textsuperscript{NCOR1\textsuperscript{AD/ID}} mice (Figure 4Ab, compare lanes 3 & 4 with 1 & 2). The quantitative band intensities are shown in Figure 4Ba, indicating a 2-fold increase in BAX and PUMA protein level. Furthermore, the increased cleaved caspase 3 (Figure 4A, panel b, lanes 3 & 4) and the decreased total poly ADP-ribose polymerase development, they were counterstained in Gill’s Hematoxylin, rinsed and mounted in Permount (Fisher Scientific, Pittsburgh, PA). The proliferative index was calculated as the percentage of Ki-67-positive nuclei to the total number of nuclei on the thyroid section. Counting was performed using National Institutes of Health (NIH) IMAGE software (ImageJ 1.34 s; Wayne Rasband, NIH, Bethesda, MD).
PARP) but increased cleaved PARP (panel c, upper band and lower band, respectively, in lanes 3 & 4) in ThrbPV/PNVcor1 ID/ID mice as compared with ThrbPV/PNVcor1 +/+ mice were indicative of the elevated apoptotic activity in thyroid tumor cells of ThrbPV/PNVcor1 ID/ID mice (also see quantitative data in Figure 4Bb).

These results indicate that in addition to delaying the G1-S cell cycle progression, the increased apoptotic activity in thyroids of ThrbPV/PNVcor1 ID/ID mice also contributed to lower thyroid growth.

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Figure 1. Expression of NCOR1ΔID increases survival of ThrbPV/PNVcor1 ID/ID mice. (A) Kaplan-Meier survival curves for ThrbPV/PNVcor1 ID/ID and ThrbPV/PNVcor1 ΔID/ΔID mice up 15 months of age. ThrbPV/PNVcor1 ID/ID (n = 29) survived significantly longer than ThrbPV/PNVcor1 +/+ mice (n = 58) (p<0.0001). (B) Thyroids of ThrbPV/PNVcor1 +/+ and ThrbPV/PNVcor1 ΔID/ΔID mice at the ages of 5–15 months were dissected and weighed. The data are presented as the ratios of thyroid weight to body weight. The differences between the thyroid weights of ThrbPV/PNVcor1 +/+ and ThrbPV/PNVcor1 ΔID/ΔID mice were significant (p<0.0001), as determined by Student’s t-test analysis.

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Figure 2. Expression of NCOR1ΔID delays thyroid cancer progression in ThrbPV/PNVcor1 ΔID/ΔID mice. (A) Hematoxylin and eosin (H&E) staining of thyroid sections (top and middle rows) and lung sections (bottom row) of ThrbPV/PNVcor1 +/+ (a, c, and e) and ThrbPV/PNVcor1 ΔID/ΔID (b, d, and f) mice. Histological sections from tissues of ThrbPV/PNVcor1 +/+ mice showed evidence of (a) vascular invasion in thyroid (arrow), (c) anaplasia in thyroid (arrows), and (e) metastatic lesions in lung (arrows). Sections of thyroids and lungs from ThrbPV/PNVcor1 ΔID/ΔID showed blood vessels (b) without vascular invasion (arrow), (d) without anaplasia, and (f) lung without metastatic lesions. (B) Comparison of age-dependent percentage occurrence of capsular invasion (a), vascular invasion (b), anaplasia (c), and lung metastasis (d). The data are expressed as the percentage of occurrence of total mutant mice examined. The symbol “#” indicates 0% occurrence.

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Inhibition of Tumor Growth by Activation of the p53-signaling Pathway in the Thyroids of ThrbPV/PVNcor1ΔID/ΔID Mice

The findings that protein abundances of p21 and BAX were altered as shown above prompted us to examine whether their expression at the mRNA level was also affected. Indeed, we found that Cdkn1A (p21WAF1) mRNA levels were significantly higher in the thyroid of ThrbPV/PVNcor1ΔID/ΔID mice than in ThrbPV/PVNcor1+/+ mice (Figure 5A, bar 2 vs. bar 1). Similarly, Bax mRNA level was higher in the thyroid of ThrbPV/PVNcor1ΔID/ΔID mice than in ThrbPV/PVNcor1+/+ mice (Figure 5B, bar 4 vs. bar 3). These two genes are directly regulated by p53 [35,36]. We therefore hypothesized that altering the activity of p53 in thyroids of ThrbPV/PVNcor1ΔID/ΔID mice would lead to activation of transcription of the Cdkn1A and Bax genes. We first examined whether the expression of p53 mRNA and protein was altered, thereby changing the activity in the thyroid of ThrbPV/PVNcor1ΔID/ΔID mice. We found that the expression of NCOR1ΔID had no effects of the expression of p53 at the mRNA level (bars 5 and 6, Figure 5A). In addition, we found that the protein abundance of p53 was similarly low in the thyroid of ThrbPV/PVNcor1ΔID/ΔID and ThrbPV/PVNcor1+/+ mice (Figure 5B, upper panel, lanes 3 and 4, respectively). Therefore, the higher Cdkn1A and Bax mRNA...
were prepared from thyroid tumors of mice (lanes 1 and 2) or ThrbPV/PV/NCOR1+ID/ID (lanes 3 and 4) mice. Shown are Western blot analyses of BAX (panel a), PUMA (panel b), cleaved caspase 3 (panel c), PARP and cleaved PARP (panel d), and actin loading control (panel e). (B) Quantitation of the band intensities shown in (A). *p < 0.05 Protein abundance of BAX and PUMA (panel a), cleaved caspase 3 and cleaved PARP (panel b) was significantly higher and total PARP (panel b) was lower in the thyroids of ThrbPV/PV+ID/+ID mice.

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activation of these two genes. The increased expression of these genes resulted in the decreased proliferation and increased apoptosis of tumor cells. These findings provided direct evidence to demonstrate that, in vivo, NCOR1 can function as an oncogene to drive thyroid cancer progression. One mechanism we uncovered is via transcription regulation by constitute association.
Therefore, NCOR1 acts in dual modes on the oncogenic actions of tumor cell proliferation and to decrease apoptosis, respectively. The repression of p53 target genes that function as tumor suppressors is therefore attenuated, and leads to cancer progression. Such oncogenic roles of NCOR1 have been demonstrated in acute promyelocyte leukemia. A fusion between the retinoic acid receptor α (RARα) and either promyelocyte leukemic (PML) or promyelocyte leukemic zinc finger (PLZF) genes sustain NCOR1 interaction. Consequently, RARα-mediated cell differentiation is blocked because of a condensed chromatin structure around the promoters of the RARα target genes [11]. However, at present we cannot exclude the possibility that NCOR1 could act via other pathways in addition to p53 signaling. Such possibilities await future exploration.

The present studies also uncovered a critical role of NCOR1 in regulating the oncogenic actions of a TR mutant, PV, via the p53 network of signaling. Previously, we showed that PV physically interacts with the C-terminal SH domain of the p85 subunit of phosphatidylinositol 3-kinase (PI3K), resulting in the activation of PI3K-AKT signaling to promote thyroid carcinogenesis of ThrbPV/PV mice [23]. Subsequently, we discovered that NCOR1, when present in the cytoplasm, could compete with PV for binding to the same C-terminal SH domain of the p85 subunit, thereby attenuating the aberrant activation of PI3K-AKT signaling by PV [39]. The present studies showed that recruitment of nuclear NCOR1/HDAC-3 by DNA-bound p53/PV attenuated the expression of two p53 target genes, Cdkn1A and Bax, to promote tumor cell proliferation and to decrease apoptosis, respectively. Therefore, NCOR1 acts in dual modes on the oncogenic actions of PV via nuclear and extranuclear actions to affect thyroid carcinogenesis of ThrbPV/PV mice. However, it is reasonable to postulate that the nuclear action could be the predominant mode of action as NCOR1 is mainly located in the nucleus. Moreover, p53, as a central node of a complex network of signaling, when bound to DNA and associated with PV/NCOR1/HDAC-3 repressor complex, could broadly attenuate the expression of an array of tumor suppressors to promote carcinogenesis. This mode of action is exemplified by the regulation of the expression of the Cdkn1A and the Bax genes (see Figure 6). Identification of additional p53 target genes affected by the recruitment of the p53/PV/NCOR1/HDAC-3 repressor complex to the p53 binding sites would reveal the breadth of the p53 network extensively affecting thyroid carcinogenesis. However, such studies are for future investigation.

Moreover, we also cannot exclude the possibility that NCOR1-ΔID could act via other pathways other than p53 signaling. NCOR1 and the homolog NCOR2/SMRT are two intensively studied co-repressors [40,41]. They both contain a conserved bipartite nuclear-receptor-interaction domain (RID) and three independent repressor domains [42]. These corepressors interact with nuclear receptors via the motif, termed the CoRNR box [43]. However, numerous biochemical studies have suggested that RAR preferentially recruits SMRT, whereas TR preferentially recruits NCOR1 [44,45], mainly because of specific sequences on the motifs as well as a TR-specific interaction domain present in NCOR1, but not SMRT [44]. Accordingly, in the present study, we used the mutant Ncor1ΔID mice in which the TR-interaction domains were deleted, thus preventing interaction with TR or PV [30]. We also focused our molecular analysis on the recruitment of the PV/NCOR1/HDAC-3 repressor complex by p53 to the p53 binding sites of target genes (Figure 5). However, at present, we cannot exclude the possibility that p53/PV could also form a PV/SMRT/HDAC-3 repressor complex, thereby contributing to thyroid carcinogenesis of ThrbPV/PV mice by repression of tumor suppressors that are downstream targets of the p53-signaling. Currently, the potential oncogenic actions of SMRT have not been extensively investigated. Whether SMRT plays a role in thyroid carcinogenesis would be interesting to address in future studies.

The present results demonstrated the amelioration of the cancer phenotypes by the expression of NCOR1ΔID in the thyroid of ThrbPV/PV mice. However, the expression of NCOR1ΔID cannot completely block the development of thyroid cancer. These
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