Induction of sodium iodide symporter gene and molecular characterisation of HNF3β/FoxA2, TTF-1 and C/EBPβ in thyroid carcinoma cells

T Akagi*,1,2,6, QT Luong1,2,6, D Gui3, J Said2, J Selektar2, A Yung1, CM Bunce4, GD Braunstein5 and HP Koeffler1,2

1Division of Hematology and Oncology, Department of Medicine, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA, USA; 2Samuel Oschin Comprehensive Cancer Center, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA, USA; 3Department of Pathology, UCLA School of Medicine, Los Angeles, CA, USA; 4School of Biosciences, University of Birmingham, Birmingham, B15 2TT, UK; 5Division of Endocrinology, Department of Medicine, Cedars-Sinai Medical Center, UCLA School of Medicine, Los Angeles, CA, USA

Thyroid carcinoma cells often do not express thyroid-specific genes including sodium iodide symporter (NIS), thyroperoxidase (TPO), thyroglobulin (TG), and thyrotropin-stimulating hormone receptor (TSHR). Treatment of thyroid carcinoma cells (four papillary and two anaplastic cell lines) with histone deacetylase inhibitors (SAHA or VPA) modestly induced the expression of the NIS gene. The promoter regions of the thyroid-specific genes contained binding sites for hepatocyte nuclear factor 3β (HNF3β)/forkhead box A2 (FoxA2), thyroid transcription factor 1 (TTF-1), and CCAAT/enhancer binding protein β (C/EBPβ). Quantitative reverse transcription-polymerase chain reaction (RT–PCR) showed decreased expression of HNF3β/FoxA2 and TTF-1 mRNA in papillary thyroid carcinoma cell lines, when compared with normal thyroid cells. Forced expression of these genes in papillary thyroid carcinoma cells inhibited their growth. Furthermore, the CpG island in the promoter region of HNF3β/FoxA2 was aberrantly methylated; and treatment with 5-aza-2-deoxycytidine (5-Aza) induced its expression. Immunohistochemical staining showed that C/EBPβ was localised in the nucleus in normal thyroid cells but was detected in the cytoplasm in papillary thyroid carcinoma cells. Subcellular fractionation of papillary thyroid carcinoma cell lines also demonstrated high levels of expression of C/EBPβ in the cytoplasm, suggesting that a large proportion of C/EBPβ protein is inappropriately localised in the cytoplasm. In summary, these findings reveal novel abnormalities in thyroid carcinoma cells.

Keywords: thyroid cancer; papillary; anaplastic; HNF3β/FoxA2; C/EBPβ

Development of thyroid carcinoma is accompanied by a block of differentiation of these cells. Papillary and follicular thyroid carcinomas initially are relatively well-differentiated tumours that over time may de-differentiate, whereas anaplastic thyroid carcinoma is an undifferentiated tumour (Farid et al., 1994). Poorly differentiated or undifferentiated carcinomas no longer express mature thyroid-specific genes including sodium iodide symporter (NIS), thyroperoxidase (TPO), thyroglobulin (TG), and thyrotropin-stimulating hormone receptor (TSHR). In normal thyroid cells, TSHR is stimulated by thyrotropin-stimulating hormone, resulting in the activation of NIS, which incorporates iodine. Thyroglobulin and iodine are catalysed into thyroid hormones by TPO (Carrasco, 1993). Investigators have predominately attempted to induce differentiation of thyroid cancer cells by exposure to compounds associated with known differentiation of other cancers.

Retinoic acid, including all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9-cis RA), induces differentiation of acute promyelocytic leukaemia cells and neuroblastoma cells and is used in the therapy for these cancers. Retinoids have been reported to induce the expression of TPO, TG, and NIS mRNAs in thyroid carcinoma cell lines (Schmutzler et al., 1997; Haugen, 2004). The NIS promoter contains CpG islands, and a DNA demethylating agent (such as, 5-aza-2-deoxycytidine (5-Aza)) combined with a histone deacetylase inhibitor has been shown to induce NIS expression and radioactive iodine uptake in follicular and anaplastic thyroid carcinoma cell lines (Venkataraman et al., 1999; Haugen, 2004).

The transcription factors of paired box gene 8 (Pax-8) and thyroid transcription factor 1 (TTF-1) have been analysed in thyroid cells. Paired box gene 8 is necessary for the formation of thyroxine-producing follicular cells in the thyroid gland (Mansouri et al., 1998, 1999); and fusion of the Pax-8 and peroxisome proliferator-activated receptor γ (PPARγ) genes occurs in approximately 30% of follicular thyroid carcinomas (Kroll et al., 2000; Dwight et al., 2003). Thyroid transcription factor 1 is required for the development of the thyroid gland, and TTF-1-deficient mice...
lack a thyroid gland and die at birth (Kimura et al., 1996). The expression of Pax-8 and TTF-1 is low in thyroid carcinoma (Ros et al., 1999); and stable transfection with a Pax-8 expression vector in anaplastic thyroid carcinoma cell line, ARO, caused re-expression of endogenous NIS, TG, and TPO (Presta et al., 2005). Reporter gene analysis found that the promoter region of TG, TPO, and TSHR could be activated by the forced expression of either Pax-8 or TTF-1 in the papillary thyroid carcinoma cell line NPA (Ros et al., 1999). Co-transfection of Pax-8 and TTF-1 restored TG promoter activity in WRO (follicular thyroid carcinoma) and ARO cells (Chun et al., 1998).

In this study, we attempted to induce differentiation of papillary and anaplastic thyroid carcinoma cells as measured by the induction of NIS, TPO, TG, and TSHR by exposing the cells to 5-Aza, histone deacetylase inhibitors (suberoylanilide hydroxamic acid (SAHA) and valproic acid), ATRA, 9-cis RA, troglitazone (PPARγ ligand), 1,25-dihydroxyvitamin D3 (1,25(OH)2D3), thyroid hormone T3, and thyrotropin-stimulating hormone. Furthermore, dysregulation of three transcription factors (TTF-1, hepatocyte nuclear factor 3 β (HNF3β)/forkhead box A2 (FoxA2), and CCAAT/enhancer binding protein β (C/EBPβ)) was examined in thyroid carcinoma cells.

**MATERIALS AND METHODS**

**Cell culture and drug treatments**

BHP (sublines 2–7, 7–13, 10–3 and 18–21) and NPA papillary, and ARO and FRO anaplastic thyroid carcinoma cell lines were cultured as described before (Fagin et al., 1996; Ohta et al., 1997; Luong et al., 2006). The normal rat thyroid cell line FRTL-5 cells (from Dr Shlomo Melmed at Cedars-Sinai Medical Center) were cultured in Ham’s F12K medium (Invitrogen, Carlsbad, CA, USA) with 5% bovine calf serum (Invitrogen) together with 10 μM 1,25(OH)2D3 (1 μM), ATRA (100 nM), 9-cis RA (100 nM), troglitazone (PPARγ ligand, 10 μM), 1,25(OH)2D3 (1 μM), thyroid hormone T3 (10 nM), and thyrotropin-stimulating hormone (1 μU ml−1) for 48–96 h. 5-Aza-2-deoxycytidine, valproic acid, thyroid hormone, and thyrotropin-stimulating hormone were dissolved in water; SAHA and troglitazone were diluted in dimethyl sulphoxide (DMSO); ATRA, 9-cis RA, and 1,25(OH)2D3 were placed in ethanol. Equal volume of DMSO and ethanol were added in control samples.

**Real-time reverse transcription polymerase chain reaction**

Total RNA was isolated from thyroid carcinoma cell lines and normal thyroid tissues using Trizol reagent (Invitrogen), and cDNA was prepared from 1 μg of total RNA with Superscript III reverse transcriptase (Invitrogen). Expression of mRNAs was measured by real-time PCR using an iCycler iQ system (Bio-Rad, Hercules, CA, USA) as described previously (Xie et al., 2001). To determine the expression levels of NIS, C/EBPα, and C/EBPβ with probes, amplification reactions were performed with the Universal Taqman PCR mastermix (Applied Biosystems, Foster City, CA, USA). The cell culture and drug treatments were performed as described before (Fagin et al., 1996; Ohta et al., 1997; Luong et al., 2006). The normal rat thyroid cell line FRTL-5 cells (from Dr Shlomo Melmed at Cedars-Sinai Medical Center) were cultured in Ham’s F12K medium (Invitrogen, Carlsbad, CA, USA) with 5% bovine calf serum (Invitrogen) together with 10 μM 1,25(OH)2D3 (1 μM), ATRA (100 nM), 9-cis RA (100 nM), troglitazone (PPARγ ligand, 10 μM), 1,25(OH)2D3 (1 μM), thyroid hormone T3 (10 nM), and thyrotropin-stimulating hormone (1 μU ml−1) for 48–96 h. 5-Aza-2-deoxycytidine, valproic acid, thyroid hormone, and thyrotropin-stimulating hormone were dissolved in water; SAHA and troglitazone were diluted in dimethyl sulphoxide (DMSO); ATRA, 9-cis RA, and 1,25(OH)2D3 were placed in ethanol. Equal volume of DMSO and ethanol were added in control samples.

**Table 1** Primer and probe sequences used for real-time RT–PCR.

| Gene name | Sequence | Melt. Temp. | Size |
|-----------|----------|------------|------|
| NIS | S AS | CCTTCATCCTGAACCAAGTG | 82.0°C | 230bp |
| TPO | S AS | ACCTCGAGGTCATTTGCA | 81.0°C | 71bp |
| TG | S AS | GTGCAACGGCATGTAAGT | 81.0°C | 87bp |
| TSHR | S AS | CCCAGCTTACCGCCCCAG | 81.0°C | 79bp |
| HNF3β/FoxA2 | S AS | AAGACCTACAGGCCACGCTA | 87.0°C | 214bp |
| TTF-1 | S AS | GCCGTACCCAGGACACGAG | 86.0°C | 265bp |
| Pax-8 | S AS | AAGTCCAGAGTGGCGCACA | 84.0°C | 331bp |
| C/EBPα | S probe | TAGAAAGATGGCTACTGGAG | — | 130bp |
| C/EBPβ | S probe | GACACAAACACAGCCAGGA | — | 102bp |
| 18S | S AS | AAACGGCTACCACTCAAGAC | 83.0°C | 155bp |
| β-actin | S AS | CCCAGATATCGTGTGAGAC | 87.0°C | 154bp |

AS, anti-sense primer; Melt. Temp., melting temperature; RT–PCR, reverse transcription-polymerase chain reaction; S, sense primer.
Radioactive iodine (\(^{125}\text{I} \)) uptake assay

\( \text{Na}^{125}\text{I} \) (100 \( \mu \text{Ci} \text{ml}^{-1} \)) stock was diluted to 0.02 \( \mu \text{Ci} \text{ml}^{-1} \) using Hanks’ Balanced Salt Solution (HBSS). Cells were plated at \( 1 \times 10^5 \) cells per well in 12-well plates and treated with appropriate drugs, either with or without 10 \( \mu \text{M} \) thyrotropin-stimulating hormone and/or cold (unlabelled) NaI. For radioactive iodine uptake, cells were washed twice with HBSS and lysed with 1 ml of 95% ethanol for 1 h at 37°C. Lysates were transferred to vials for counting, and total counts were normalised to number of viable cells in parallel cultures.

Plasmid transfection and colony assay

Expression vectors of \( \text{pTTF-1} \) and \( \text{pHNF3} / \text{FoxA2} \) were generous gifts from Dr Edward Morrisey (University of Pennsylvania). Plasmids of 1 \( \mu \text{g} \) \( \text{pTag1} \) (empty vector), \( \text{pTTF-1} \), and \( \text{pHNF3} / \text{FoxA2} \) were transfected into BHPs cells using Lipofectamine 2000 (Invitrogen). The zinc-inducible \( \text{pMT-C/EBP} / \text{FoxA2} \) were transfected into BHPs cells using Lipofectamine 2000 (Invitrogen). The zinc-inducible \( \text{pMT-C/EBP} / \text{FoxA2} \) expression plasmid (Gery et al, 2005) was transfected into BHP cells (sublines 2–7 and 7–13), which were selected with 500 \( \mu \text{g} \text{ml}^{-1} \) G418 for 48 h.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and clonogenic soft agar assay

Cells were treated with 10 \( \mu \text{l} \) of 5 \( \text{mg ml}^{-1} \) MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St Louis, MO, USA), and incubated at 37°C for 4 h. Medium was removed, and 50 \( \mu \text{l} \) DMSO was added to the cells to solubilise the MTT. Plates were read at wavelength of 540 nm on a plate reader.

For clonogenic soft agar assays, cells were plated into 24-well flat-bottomed wells using a two-layer soft agar system with 1 \( \times 10^5 \) cells per well in a volume of 400 \( \mu \text{l} \) per well as previously described (Luong et al, 2006). After 14 days of incubation, colonies were counted.

Methylation analysis of \( \text{HNF3} / \text{FoxA2} \) gene

Genomic DNA was modified by sodium bisulphate using EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA). The Cpg island (−761 to −561, ATG codon considered as +1) of the \( \text{HNF3} / \text{FoxA2} \) gene was amplified from the bisulphate-modified genomic DNA with specific primers (sense primer: 5' TTGTTTGGTTG-3', anti-sense primer: 5'-AAATAATCAACTCAG ACC-3'). For PCR amplification, a total volume of 10 \( \mu \text{l} \) was used containing modified genomic DNA, 0.5 \( \mu \text{M} \) of each primers, 50 \( \mu \text{l} \) of FailSafe PCR 2 x PreMixe E (Epicentre Biotechnologies, Madison, WI, USA) and 1.0 U platinum Taq (Invitrogen).

For colony assay, cells transfected with plasmid were plated at \( 1 \times 10^5 \) cells per well in 12-well plates in 1 ml culture media containing 500 \( \mu \text{g} \text{ml}^{-1} \) G418. After 2 weeks, cells were stained with Crystal violet dye (0.25% crystal violet dissolved in 50% methanol).

Relative expression of NIS mRNA

For colony assay, cells transfected with plasmid were plated at \( 1 \times 10^5 \) cells per well in 12-well plates in 1 ml culture media containing 500 \( \mu \text{g} \text{ml}^{-1} \) G418. After 2 weeks, cells were stained with Crystal violet dye (0.25% crystal violet dissolved in 50% methanol).

For colony assay, cells transfected with plasmid were plated at \( 1 \times 10^5 \) cells per well in 12-well plates in 1 ml culture media containing 500 \( \mu \text{g} \text{ml}^{-1} \) G418. After 2 weeks, cells were stained with Crystal violet dye (0.25% crystal violet dissolved in 50% methanol).

For colony assay, cells transfected with plasmid were plated at \( 1 \times 10^5 \) cells per well in 12-well plates in 1 ml culture media containing 500 \( \mu \text{g} \text{ml}^{-1} \) G418. After 2 weeks, cells were stained with Crystal violet dye (0.25% crystal violet dissolved in 50% methanol).

For colony assay, cells transfected with plasmid were plated at \( 1 \times 10^5 \) cells per well in 12-well plates in 1 ml culture media containing 500 \( \mu \text{g} \text{ml}^{-1} \) G418. After 2 weeks, cells were stained with Crystal violet dye (0.25% crystal violet dissolved in 50% methanol).

For colony assay, cells transfected with plasmid were plated at \( 1 \times 10^5 \) cells per well in 12-well plates in 1 ml culture media containing 500 \( \mu \text{g} \text{ml}^{-1} \) G418. After 2 weeks, cells were stained with Crystal violet dye (0.25% crystal violet dissolved in 50% methanol).
Polymerase chain reaction products were subcloned into pCR 2.1 vector (Invitrogen) and sequenced.

Subcellular fractionation, Western blot analysis, and immunohistochemistry

Total cell lysates were prepared by lysing cells in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.5)) containing a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) as well as 1 mM NaF and 1 mM NaVO4. To separate nuclear and cytoplasmic fractions, cells were fractionated with NE-PER Nuclear and Cytoplasmic Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA). These samples were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by an electrotransfer to polyvinylidene difluoride membrane. The signals were developed with either Supersignal West Pico Chemiluminescent or Supersignal West Dura Extended Duration Substrate (Pierce Biotechnology). Anti-PPARγ, C/EBPβ, β-actin, and heterogeneous nuclear ribonuclear protein (hnRNP) A1 antibodies were obtained from Santa Cruz Technology (Santa Cruz, CA, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Research Diagnostics Inc. (Concord, MA, USA).

Normal and papillary thyroid carcinoma tissue blocks were cut at 3 μM thickness, deparaffinised and pre-treated in Tris-HCl (pH 9.0). These samples were incubated overnight with anti-C/EBPβ antibody (1:500 dilution), washed, followed by the HRP-conjugated secondary antibody (Dako, Carpinteria, CA, USA), and DAB chromogen. The tissues were counterstained with haematoxylin and then coverslipped. Three samples of normal and three carcinomas were examined.

RESULTS

Induction of expression of thyroid-specific genes: effect of 5-Az, SAHA, valproic acid, and nuclear hormone receptor ligands in thyroid carcinoma cell lines

Silencing of genes can be associated with epigenetic change including abnormal methylation of CpG islands and/or deacetylation of histones. Therefore, papillary (BHP sublines 2–7, 7–13, 10–3, and 18–21) and two anaplastic thyroid carcinoma (ARO and FRO) cell lines were cultured either with or without 5-Az (1 μM) and/or histone deacetylase inhibitors (SAHA (5 μM) or valproic acid (1 μM)). Quantitative reverse transcription-polymerase chain reaction (RT–PCR) showed that expressions of thyroid-specific genes (TPO, TG, and TSHR) were either extremely low or at undetectable levels compared with normal thyroid tissue (data not shown). We also examined the effects of several ligands of nuclear hormone receptors either with or without SAHA. As shown in

Figure 2  Expression of transcription factors in normal thyroid cells and thyroid carcinoma cell lines. Expression of transcription factors including HNF3β/FoxA2, TTF-1, Pax-8, C/EBPα, and C/EBPβ was measured by quantitative RT–PCR in five normal thyroid samples and five thyroid carcinoma cell lines (BHPs 2–7, 7–13, 18–21, ARO, and FRO). Expression in individual samples or cell lines is represented by open circles, mean expression of normal or cancer cells is represented by the horizontal bar ± s.e.
Thyroid-related transcription factors are poorly expressed in papillary and anaplastic thyroid carcinoma cell lines

The above data showed that the four thyroid-specific genes (NIS, TPO, TG, and TSHR) were negligibly expressed in the thyroid carcinoma cell lines; therefore, common transcription factor(s) that regulate these genes might not be expressed in these cell lines. The promoter regions of these genes contain putative transcription factor binding sites for HNF3β/FoxA2, C/EBPs, PPARγ, Pax-8, and TTF-1. Real-time PCR showed that HNF3β/FoxA2, TTF-1, and Pax-8 were expressed in normal thyroid tissue; in contrast, levels were either very low or undetectable in thyroid carcinoma cell lines (Figure 2). Western blot analysis showed that the protein expression of PPARγ was barely detectable in BHP sublines, but easily found in ARO and FRO cell lines (data not shown).

Forced expression of either HNF3β/FoxA2 or TTF-1 in thyroid carcinoma cells

Next, we transfected either the HNF3β/FoxA2 or the TTF-1 expression vector into papillary thyroid carcinoma cell lines. Neither HNF3β/FoxA2- nor TTF-1-expressing BHP cells (subline 2–7) had an increase in 125I uptake, when compared with normal FRTL-5 thyroid cells (Figure 3). Nevertheless, the forced expression of either HNF3β/FoxA2 or TTF-1 resulted in growth inhibition compared with cells transfected with an empty vector as measured by MTT assay (data not shown), suggesting that these transcription factors have antiproliferative activity in papillary thyroid carcinoma cells.

Methylation status of HNF3β/FoxA2 gene in papillary thyroid carcinoma cells

The HNF3β/FoxA2 gene has a CpG island in its promoter; and the region is often methylated in breast and lung cancers (Halmos et al, 2004; Miyamoto et al, 2005) prompting us to examine thyroid carcinoma cells. The great majority of the 21 CpG sites in the promoter were methylated in BHP (subline 2–7) and NPA papillary thyroid carcinoma cells (Figure 4A), as well as in the anaplastic thyroid carcinoma cells. The promoter regions of these genes contain putative transcription factor binding sites for HNF3β/FoxA2, C/EBPs, PPARγ, Pax-8, and TTF-1. Real-time PCR showed that HNF3β/FoxA2, TTF-1, and Pax-8 were expressed in normal thyroid tissue; in contrast, levels were either very low or undetectable in thyroid carcinoma cell lines (Figure 2). Western blot analysis showed that the protein expression of PPARγ was barely detectable in BHP sublines, but easily found in ARO and FRO cell lines (data not shown).

Forced expression of either HNF3β/FoxA2 or TTF-1 in thyroid carcinoma cells

Next, we transfected either the HNF3β/FoxA2 or the TTF-1 expression vector into papillary thyroid carcinoma cell lines. Neither HNF3β/FoxA2- nor TTF-1-expressing BHP cells (subline 2–7) had an increase in 125I uptake, when compared with normal FRTL-5 thyroid cells (Figure 3). Nevertheless, the forced expression of either HNF3β/FoxA2 or TTF-1 resulted in growth inhibition compared with cells transfected with an empty vector as measured by MTT assay (data not shown), suggesting that these transcription factors have antiproliferative activity in papillary thyroid carcinoma cells.

Methylation status of HNF3β/FoxA2 gene in papillary thyroid carcinoma cells

The HNF3β/FoxA2 gene has a CpG island in its promoter; and the region is often methylated in breast and lung cancers (Halmos et al, 2004; Miyamoto et al, 2005) prompting us to examine thyroid carcinoma cells. The great majority of the 21 CpG sites in the promoter were methylated in BHP (subline 2–7) and NPA papillary thyroid carcinoma cells (Figure 4A), as well as in the anaplastic thyroid carcinoma cells. The promoter regions of these genes contain putative transcription factor binding sites for HNF3β/FoxA2, C/EBPs, PPARγ, Pax-8, and TTF-1. Real-time PCR showed that HNF3β/FoxA2, TTF-1, and Pax-8 were expressed in normal thyroid tissue; in contrast, levels were either very low or undetectable in thyroid carcinoma cell lines (Figure 2). Western blot analysis showed that the protein expression of PPARγ was barely detectable in BHP sublines, but easily found in ARO and FRO cell lines (data not shown).

Forced expression of either HNF3β/FoxA2 or TTF-1 in thyroid carcinoma cells

Next, we transfected either the HNF3β/FoxA2 or the TTF-1 expression vector into papillary thyroid carcinoma cell lines. Neither HNF3β/FoxA2- nor TTF-1-expressing BHP cells (subline 2–7) had an increase in 125I uptake, when compared with normal FRTL-5 thyroid cells (Figure 3). Nevertheless, the forced expression of either HNF3β/FoxA2 or TTF-1 resulted in growth inhibition compared with cells transfected with an empty vector as measured by MTT assay (data not shown), suggesting that these transcription factors have antiproliferative activity in papillary thyroid carcinoma cells.

Methylation status of HNF3β/FoxA2 gene in papillary thyroid carcinoma cells

The HNF3β/FoxA2 gene has a CpG island in its promoter; and the region is often methylated in breast and lung cancers (Halmos et al, 2004; Miyamoto et al, 2005) prompting us to examine thyroid carcinoma cells. The great majority of the 21 CpG sites in the promoter were methylated in BHP (subline 2–7) and NPA papillary thyroid carcinoma cells (Figure 4A), as well as in the anaplastic thyroid carcinoma cells. The promoter regions of these genes contain putative transcription factor binding sites for HNF3β/FoxA2, C/EBPs, PPARγ, Pax-8, and TTF-1. Real-time PCR showed that HNF3β/FoxA2, TTF-1, and Pax-8 were expressed in normal thyroid tissue; in contrast, levels were either very low or undetectable in thyroid carcinoma cell lines (Figure 2). Western blot analysis showed that the protein expression of PPARγ was barely detectable in BHP sublines, but easily found in ARO and FRO cell lines (data not shown).

Forced expression of either HNF3β/FoxA2 or TTF-1 in thyroid carcinoma cells

Next, we transfected either the HNF3β/FoxA2 or the TTF-1 expression vector into papillary thyroid carcinoma cell lines. Neither HNF3β/FoxA2- nor TTF-1-expressing BHP cells (subline 2–7) had an increase in 125I uptake, when compared with normal FRTL-5 thyroid cells (Figure 3). Nevertheless, the forced expression of either HNF3β/FoxA2 or TTF-1 resulted in growth inhibition compared with cells transfected with an empty vector as measured by MTT assay (data not shown), suggesting that these transcription factors have antiproliferative activity in papillary thyroid carcinoma cells.

Methylation status of HNF3β/FoxA2 gene in papillary thyroid carcinoma cells

The HNF3β/FoxA2 gene has a CpG island in its promoter; and the region is often methylated in breast and lung cancers (Halmos et al, 2004; Miyamoto et al, 2005) prompting us to examine thyroid carcinoma cells. The great majority of the 21 CpG sites in the promoter were methylated in BHP (subline 2–7) and NPA papillary thyroid carcinoma cells (Figure 4A), as well as in the anaplastic thyroid carcinoma cells. The promoter regions of these genes contain putative transcription factor binding sites for HNF3β/FoxA2, C/EBPs, PPARγ, Pax-8, and TTF-1. Real-time PCR showed that HNF3β/FoxA2, TTF-1, and Pax-8 were expressed in normal thyroid tissue; in contrast, levels were either very low or undetectable in thyroid carcinoma cell lines (Figure 2). Western blot analysis showed that the protein expression of PPARγ was barely detectable in BHP sublines, but easily found in ARO and FRO cell lines (data not shown).

Forced expression of either HNF3β/FoxA2 or TTF-1 in thyroid carcinoma cells

Next, we transfected either the HNF3β/FoxA2 or the TTF-1 expression vector into papillary thyroid carcinoma cell lines. Neither HNF3β/FoxA2- nor TTF-1-expressing BHP cells (subline 2–7) had an increase in 125I uptake, when compared with normal FRTL-5 thyroid cells (Figure 3). Nevertheless, the forced expression of either HNF3β/FoxA2 or TTF-1 resulted in growth inhibition compared with cells transfected with an empty vector as measured by MTT assay (data not shown), suggesting that these transcription factors have antiproliferative activity in papillary thyroid carcinoma cells.

Methylation status of HNF3β/FoxA2 gene in papillary thyroid carcinoma cells

The HNF3β/FoxA2 gene has a CpG island in its promoter; and the region is often methylated in breast and lung cancers (Halmos et al, 2004; Miyamoto et al, 2005) prompting us to examine thyroid carcinoma cells. The great majority of the 21 CpG sites in the promoter were methylated in BHP (subline 2–7) and NPA papillary thyroid carcinoma cells (Figure 4A), as well as in the anaplastic thyroid carcinoma cells. The promoter regions of these genes contain putative transcription factor binding sites for HNF3β/FoxA2, C/EBPs, PPARγ, Pax-8, and TTF-1. Real-time PCR showed that HNF3β/FoxA2, TTF-1, and Pax-8 were expressed in normal thyroid tissue; in contrast, levels were either very low or undetectable in thyroid carcinoma cell lines (Figure 2). Western blot analysis showed that the protein expression of PPARγ was barely detectable in BHP sublines, but easily found in ARO and FRO cell lines (data not shown).

Forced expression of either HNF3β/FoxA2 or TTF-1 in thyroid carcinoma cells

Next, we transfected either the HNF3β/FoxA2 or the TTF-1 expression vector into papillary thyroid carcinoma cell lines. Neither HNF3β/FoxA2- nor TTF-1-expressing BHP cells (subline 2–7) had an increase in 125I uptake, when compared with normal FRTL-5 thyroid cells (Figure 3). Nevertheless, the forced expression of either HNF3β/FoxA2 or TTF-1 resulted in growth inhibition compared with cells transfected with an empty vector as measured by MTT assay (data not shown), suggesting that these transcription factors have antiproliferative activity in papillary thyroid carcinoma cells.
thyroid carcinoma cell line FRO (data not shown). In contrast, the region was unmethylated in normal thyroid tissues (Figure 4B). Real-time PCR showed that the expression of HNF3β/FoxA2 mRNA was induced after the treatment of BHP cells (subline 2–7) with the demethylating agent 5-Az (1 μM, 96 h) (Figure 4C). Taken together, these results suggest that the expression of the HNF3β/FoxA2 gene is epigenetically repressed in thyroid carcinoma cell lines.

**Forced expression of C/EBPβ in thyroid carcinoma cells**

Next, we placed a Zn-inducible C/EBPβ expression vector into BHP cells (sublines 2–7 and 7–13) (Figure 5A). CCAAT/enhancer binding protein β has two isoforms, LAP (liver-enriched transcriptional activating protein) and LIP (liver-enriched transcriptional inhibitory protein). The smaller form of C/EBPβ (LIP) clearly increased in the cells treated with zinc. Induction of C/EBPβ expression resulted in a 60% growth reduction compared to the non-induced cells (Figure 5B, left panel). The more sensitive clonogenic soft agar assay showed that clonogenic growth decreased in the absence of zinc. Nevertheless, clonogenic growth decreased 50% in the absence of zinc (Figure 5B, right panel). Similarly, crystal violet staining demonstrated that C/EBPβ had anti-growth activity in another subline, BHP 17-3 (Figure 5C). Taken together, these results suggested that forced expression of C/EBPβ can cause growth inhibition in BHP papillary thyroid carcinoma cells.

**Cellular localisation of C/EBPβ in human normal and papillary thyroid carcinoma tissues and cell lines**

To examine expression of C/EBPβ in human thyroid tissues, normal and papillary thyroid carcinoma tissues were stained with anti-C/EBPβ antibody. Immunohistochemistry revealed that C/EBPβ signal was strongly detected in the nucleus in normal thyroid cells (Figure 6A). Interestingly, C/EBPβ was detected in the cytoplasm and to a lesser extent the nucleus of papillary thyroid carcinoma cells (Figure 6B). The subcellular localisation of C/EBPβ in four thyroid carcinoma cell lines (BHP2-7, NPA, FRO, and ARO) was also determined by fractionation (Figure 6C). CCAAT/enhancer binding protein β-LAP was detected in both the nucleus and the cytoplasm. CCAAT/enhancer binding protein β-LIP, which has a dominant-negative activity against LAP, was expressed in NPA and FRO cell lines, and localised in the nucleus.

**DISCUSSION**

We attempted to induce differentiation and inhibit proliferation of thyroid carcinoma cells with various compounds and transcription factors; and in addition, we explored the abnormalities in endogenous expression of these transcription factors in thyroid carcinoma cells. Suberoylanilide hydroxamic acid modestly induced the expression of TPO, TG, and TSHR and the combination of SAHA and 1,25(OH)2D3 further enhanced the expression of NIS in BHP cell line. However, these agents were not potent stimulators of NIS expression level, when compared with expression levels found in normal thyroid tissues. Induced expression of these transcripts was about 10- to 100-fold lower than those found in normal thyroid cells. Therefore taken together, our data suggest that these compounds had little differentiation inducing activity and would be unlikely candidates to enhance the therapeutic value of radioactive iodine (131I) for the treatment of thyroid tumours. Notably, two histone deacetylase inhibitors, depsipeptide and Trichostatin A, have been shown to induce the expression of NIS and 125I uptake in several follicular and anaplastic thyroid carcinoma cell lines (Haugen, 2004).
Survey of the thyroid-specific genes showed that each promoter had transcription factor binding sites for HNF3β/FoxA2, TTF-1, C/EBPβ, and Pax-8. Earlier studies using either the TG or TPO promoter found that they were activated by TTF-1 and HNF3β/FoxA2 in thyroid carcinoma cell lines (Sato and Di Lauro, 1996; Ros et al, 1999; Shimura et al, 2001). In addition, Pax-8 leads to the re-expression of NIS, TPO, TG, and TTF-1 mRNAs in ARO cells (Presta et al, 2005). Our present study demonstrated that forced expression of either HNF3β/FoxA2 or TTF-1 was unable to induce differentiation of the thyroid cancer cells as measured by NIS mRNA expression and radiiodine uptake. Similarly, co-transfection of HNF3β/FoxA2 and TTF-1 did not induce the expression of TPO, TG or TSHR mRNAs in BHP cells (data not shown), indicating that other molecule(s) might be required to induce endogenous mRNA expression of these thyroid-related differentiation genes. Interestingly, HNF3β/FoxA2 is a methylated gene in breast and lung cancer cells; and overexpression of HNF3β/FoxA2 in a lung cancer cell line leads to growth arrest and apoptosis (Halmos et al, 2004; Miyamoto et al, 2005). Here, we report for the first time that the presence of aberrant methylation of HNF3β/FoxA2 in thyroid carcinoma cell lines, and forced expression of the gene, resulted in growth inhibition.

Recently, Pomerance et al (2005) detected cytoplasmic localisation of C/EBPβ in papillary thyroid carcinoma tissues. Our immunohistochemical analysis also showed cytoplasmic localisation of C/EBPβ in papillary thyroid carcinoma tissues. In addition, we demonstrated by cell fractionation that C/EBPβ-LAP was present in both the nucleus and the cytoplasm; in contrast, C/EBPβ-LIP, a dominant-negative form of C/EBPβ, was localised in the nucleus in NPA and FRO cells. Nucleocyttoplasmic distribution of C/EBPβ has been found in several other types of cancer. Human acute myeloid leukaemic cell line HL-60 showed cytoplasmic localisation of C/EBPβ when Thr235 of C/EBPβ-LAP was phosphorylated, and the induction of differentiation and the inhibition of proliferation of these cells by 1,25(OH)2D3 resulted in nuclear translocation of the transcription factor (Marcinkowska et al, 2006). In other experiments, C/EBPβ phosphorylation at Ser288 was associated with cytoplasmic localisation of the protein in human liver cancer cells; in contrast, normal liver cells had neither phosphorylation of Ser288 nor cytoplasmic C/EBPβ (Buck et al, 2001). CCAAT/enhancer binding protein β-LAP and -LIP contain both nuclear localisation signal and nuclear export signal in their common C-terminal region (Williams et al, 1997). The N-terminal region, which is specific for C/EBPβ-LAP, might contain a motif that causes cytoplasmic retention in thyroid cancer cells. In general, transcription factors including C/EBPβ function in the nucleus, suggesting that deregulation of nuclear localisation of C/EBPβ leads to functional deficiency and result in cell abnormalities.

In summary, we found that the thyroid cancer cells had decreased the expression of TTF-1 and HNF3β/FoxA2; and their forced re-expression was associated with decreased cell growth. In addition, methylation of HNF3β/FoxA2 and inappropriate cellular localisation of C/EBPβ were identified as novel abnormalities. Future studies will screen for small molecules that can induce expression of these transcription factors resulting in a unique therapy for thyroid cancer.

**Figure 6** Cellular localisation of C/EBPβ by immunohistochemistry in normal and papillary thyroid carcinoma tissues. Normal thyroid (A) and papillary thyroid carcinoma (B) tissues were immunohistochemically stained with anti-C/EBPβ antibody. Photomicrographs are representative of three different samples of both normal thyroid and papillary thyroid carcinoma (data not shown). (C) Cellular fractionation of C/EBPβ in papillary and anaplastic thyroid carcinoma cells. Papillary (BHP subline 2–7 and NPA) and anaplastic thyroid carcinoma (FRO and ARO) cells were fractionated into the nuclear and cytoplasmic lysates, and the localisation of C/EBPβ was determined by electrophoresis followed by Western blot analysis. Glyceraldehyde-3-phosphate dehydrogenase and hnRNP A1 are cytoplasm and nuclear markers, respectively. N, nuclear fraction; C, cytoplasmic fraction; LAP, liver-enriched transcriptional activating protein; LIP, liver-enriched transcriptional inhibitory protein.
ACKNOWLEDGEMENTS

We are grateful to Drs Shlomo Melmed and Edward Morrissey for gifts of FRTL-5 cell lines and the pTTF-1 and pHNF3/FoxA2 plasmids, respectively. We thank Drs Adrian F Gombart and Sigal Gery for helpful discussions. We acknowledge the generous support of the Inger Foundation, and the C & H Koeffler Fund. GDB is holder of the James R Klinenberg, MD, Chair in Medicine. HPK is the holder of the Mark Goodson Chair in Oncology Research at Cedars-Sinai Medical Center, and is a member of the Jonsson Cancer Center and the Molecular Biology Institute at UCLA.

REFERENCES

Buck M, Zhang L, Halasz NA, Hunter T, Chojkier M (2001) Nuclear export of phosphorylated C/EBPbeta mediates the inhibition of albumin expression by TNF-alpha. *EMBO J* 20: 6712–6723

Carrasco N (1993) Iodide transport in the thyroid gland. *Biochem Biophys Acta* 1154: 65 – 82

Chun YS, Saji M, Zeiger MA (1998) Overexpression of TTF-1 and PAX-8 restores thyroglobulin gene promoter activity in ARO and WRO cell lines. *Surgery* 124: 1100 – 1105

Dwight T, Hoppe SR, Foukakis T, Lui WO, Wallin G, Höög A, Frisk T, Larsson C, Zedenius J (2003) Involvement of the PAX8/peroxisome proliferator-activated receptor gamma rearrangement in follicular thyroid tumors. *J Clin Endocrin Metab* 88: 4440 – 4445

Farid NR, Shi Y, Zou M (1994) Molecular basis of thyroid cancer. *Endocr Rev* 15: 202 – 232

Fagin JA, Tang SH, Zeki K, Di Lauro R, Fusco A, Gonsky R (1996) Expression, hormonal regulation, and subcellular localization of CCAAT/ enhancer-binding protein-beta in rat and human thyrocytes. *Thyroid* 15: 197 – 204

Fletcher JA (2000) PAX8-PPAR-gamma-1 fusion in oncogene human thyroid carcinoma cell line by introduction of wild-type p53. *Cancer Res* 56: 765 – 771

Gery S, Gombart AF, Yi WS, Koeffler C, Hofmann WK, Koeffler HP (2005) Expression by TNF-alpha. *Endocur Biophys Res Commun* 2005: 4137 – 4147

Haugen BR (2004) Redifferentiation therapy in advanced thyroid cancer. *Curr Drug Targets Immune Endocr Metabol Disord* 4: 175 – 180

Kimura S, Hara Y, Pineau T, Fernandez-Salgueiro P, Fox CH, Ward JM, Gonzalez FJ (1996) The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev* 10: 60 – 69

Kroll TG, Sarraf P, Peciarini L, Chen CJ, Mueller E, Spiegelman BM, Fletcher JA (2000) PAX8-PPAR-gamma-1 fusion in oncogene human thyroid carcinoma. *Science* 289: 1357 – 1360

Luong QT, O’Kelly J, Braunstein GD, Hershman JM, Koeffler HP (2006) Antitumor activity of suberylanilide hydroxamic acid against thyroid cancer cell lines in vitro and in vivo. *Clin Cancer Res* 12: 5570 – 5577

Mansouri A, Chowdhury K, Gruss P (1998) Follicular cells of the thyroid gland require Pax8 gene function. *Nature Genet* 19: 87 – 90

Mansouri A, St-Onge L, Gruss P (1999) Role of Pax genes in endoderm-derived organs. *Trends Endocrinol Metab* 10: 164 – 167

Marcinkowska E, Garay E, Gocek E, Chrobak A, Wang X, Studzinski GP (2006) Regulation of C/EBPbeta isoforms by MAPK pathways in HL60 cells induced to differentiate by 1,25-dihydroxyvitamin D3. *Exp Cell Res* 312: 2054 – 2065

Miyamoto K, Fukutomi T, Akashi-Tanaka S, Hasegawa T, Asahara T, Sugimura T, Ushijima T (2005) Identification of 20 genes aberrantly methylated in human breast cancers. *Int J Cancer* 116: 407 – 414

Ohta K, Pang XP, Berg L, Hershman JM (1997) Growth inhibition of new human thyroid carcinoma cell lines by adenylate cyclase through the beta-adrenergic receptor. *J Clin Endocrinol Metab* 82: 2633 – 2638

Pomérance M, Mockey M, Young J, Quillard J, Blondue JP (2005) Expression, hormonal regulation, and subcellular localization of CCAAT/ enhancer-binding protein-beta in rat and human thyrocytes. *Thyroid* 15: 413 – 419

Presta I, Arturi F, Ferretti E, Mattei T, Scarpetti D, Tosi E, Scipioni A, Celano M, Gulino A, Filetti S, Russo D (2005) Recovery of NIS expression in thyroid cancer cells by overexpression of Pax8 gene. *BMC Cancer* 5: 80

Ros P, Rossi DL, Acebron A, Santisteban P (1999) Thyroid-specific gene expression in the multi-step process of thyroid carcinogenesis. *Biochimie* 81: 389 – 396

Sato K, Di Lauro R (1996) Hepatocyte nuclear factor 3beta participates in the transcriptional regulation of the thyroperoxidase promoter. *Biochem Biophys Res Commun* 220: 86 – 93

Schmutzler C, Winzer R, Meiessner-Weigl J, Köhle J (1997) Retinoic acid increases sodium/iodide symporter mRNA levels in human thyroid cancer cell lines and suppresses expression of functional symporter in nontransformed FRTL-5 rat thyroid cells. *Biochem Biophys Res Commun* 240: 832 – 838

Shimura H, Suzuki H, Miyazaki A, Furuya F, Ohta K, Haraguchi K, Endo T, Onaya T (2001) Transcriptional activation of the thyroglobulin promoter directing suicide gene expression by thyroid transcription factor-1 in thyroid cancer cells. *Cancer Res* 61: 3640 – 3646

Venkataraman GM, Yatin M, Marcinkewicz R, Ain KB (1999) Preservation of iodide uptake in differentiated thyroid carcinoma: relationship to human Na+/I– symporter gene methylation status. *Clin Endocrinol Metab* 84: 2449 – 2457

Williams SG, Angerer ND, Johnson PF (1997) C/EBP proteins contain nuclear localization signals imbedded in their basic regions. *Gene Expr* 6: 371 – 385

Xie D, Nakachi K, Wang H, Elshoff R, Koeffler HP (2001) Elevated levels of connective tissue growth factor, WISP-1, and CYF61 in primary breast cancers associated with more advanced features. *Cancer Res* 61: 8917 – 8923