5-Aza-2'-Deoxycytidine Has Minor Effects on Differentiation in Human Thyroid Cancer Cell Lines, But Modulates Genes That Are Involved in Adaptation *In Vitro*

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Background: In thyroid cancer, the lack of response to specific treatment, for example, radioactive iodine, can be caused by a loss of differentiation characteristics of tumor cells. It is hypothesized that this loss is due to epigenetic modifications. Therefore, drugs releasing epigenetic repression have been proposed to reverse this silencing.

Methods: We investigated which genes were reinduced in dedifferentiated human thyroid cancer cell lines when treated with the demethylating agent 5-aza-2'-deoxycytidine (5-Aza-dC) and the histone deacetylase inhibitors trichostatin A (TSA) and suberoylanilide hydroxamic acid, by using reverse transcriptase–polymerase chain reaction and microarrays. These results were compared to the expression patterns in *in vitro* human differentiated thyrocytes and in *in vivo* dedifferentiated thyroid cancers. In addition, the effects of 5-Aza-dC on DNA quantities and cell viability were investigated.

Results: Among the canonical thyroid differentiation markers, most were not, or only to a minor extent, re-expressed by 5-Aza-dC, whether or not combined with TSA or forskolin, an inducer of differentiation in normal thyrocytes. Furthermore, 5-Aza-dC–modulated overall mRNA expression profiles showed only few commonly regulated genes compared to differentiated cultured primary thyrocytes. In addition, most of the commonly strongly 5-Aza-dC–induced genes in cell lines were either not regulated or upregulated in anaplastic thyroid carcinomas. Further analysis of which genes were induced by 5-Aza-dC showed that they were involved in pathways such as apoptosis, antigen presentation, defense response, and cell migration. A number of these genes had similar expression responses in 5-Aza-dC–treated nonthyroid cell lines.

Conclusions: Our results suggest that 5-Aza-dC is not a strong inducer of differentiation in thyroid cancer cell lines. Under the studied conditions and with the model used, 5-Aza-dC treatment does not appear to be a potential redifferentiation treatment for dedifferentiated thyroid cancer. However, this may reflect primarily the inadequacy of the model rather than that of the treatment. Moreover, the observation that 5-Aza-dC negatively affected cell viability in cell lines could still suggest a therapeutic opportunity. Some of the genes that were modulated by 5-Aza-dC were also induced in nonthyroid cancer cell lines, which might be explained by an epigenetic modification resulting in the adaptation of the cell lines to their culture conditions.

Introduction

A LOSS OF DIFFERENTIATION, during which cells gradually lose the expression of their organ-specific tissue characteristics, is a part of the process of cancer progression. This is often accompanied by a lack of response to target-specific treatments. Thyroid neoplasms are an interesting model to

study differentiation and dedifferentiation processes, because they include a spectrum of different morphologically recognizable grades of malignancy and levels of expression of differentiation markers. Although differentiated thyroid cancers offer good treatment opportunities, poorly differentiated and dedifferentiated thyroid cancers (e.g., anaplastic thyroid carcinomas [ATC]) still remain an important clinical challenge,

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with most patients with ATC dying within 6 months (1–5). Unresponsive thyroid cancers have lost the functional expression of the sodium iodide symporter (NIS or SLC5A5), the first protein involved in the synthesis of thyroid hormones (6). As radioactive iodine (RAI) uptake is mediated by this symporter, the absence of NIS precludes its use to detect and treat such cancers. Therefore, any (even short) treatment transiently inducing the expression of functional NIS protein and thus RAI uptake by the tumor would give a window of opportunity for a potentially curative treatment.

Epigenetic alterations are a common finding in thyroid tumors (7), and epigenetic silencing of a number of genes has been reported, including hypermethylation of thyroid differentiation genes such as the thyrotropin receptor (*TSHR*) (7–10) and *NIS* (10,11). Based on these observations, re-expression of these hypermethylated genes might result after treatment with demethylating agents or other chromatin-modifying drugs such as inhibitors of histone deacetylases (HDAC). A first study investigating the effect of the DNA methylation inhibitor 5-azacytidine on NIS expression in cell lines reported an increase of *NIS* mRNA expression in four out of seven tumor cell lines after treatment, but an increased iodide transport was detected only in two of them (12). However, these cell lines were later shown to be of nonthyroid origin (13). Other studies have also investigated epigenetic treatments of thyroid cancer cell lines on differentiation, and although modulations of thyroid-specific genes have been described, this did not always lead to functional NIS expression in all of the investigated cell lines (14–23). To investigate whether culture conditions might influence the expression of thyroid-specific genes, we analyzed the effect of the DNA methylation inhibitor 5-aza-2'-deoxycytidine (5-AzadC, decitabine), for which a dual action has been reported: it reactivates the silenced genes, and it induces differentiation at low doses, and it is cytotoxic at high doses (24). Previously, we showed that thyroid cancer cell lines from different origins have lost the expression of most classical thyroid differentiation markers and that, compared to *in vivo* thyroid tumors, regardless of their origin, their gene expression profiles were closest to ATC (25,26). Therefore, we used these cell lines as a model for dedifferentiated thyroid cancers and asked which genes could be reinduced by treatment with 5-AzadC alone or in combination with other agents. These combinations included HDAC inhibitors such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), and the adenylate cyclase activator forskolin, the latter being a stimulator of differentiation in normal thyrocytes. Compounds were tested using various concentrations, drug combinations, treatment times, and different culture conditions. The effect of drug treatments was evaluated by studying the expression of a panel of differentiation genes by quantitative and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and investigating drug-induced gene expression profiles by microarray analysis. 5-AzadC-modulated profiles were also compared to expression levels in differentiated primary thyrocytes *in vitro*, in ATC *in vivo*, and in 5-AzadC-treated nonthyroid cell lines. In addition, the effect of 5-AzadC on cell growth was investigated.

Materials and Methods

Cell lines

Human thyroid cancer cell lines were originally derived from follicular thyroid carcinomas (FTC133 and WRO), papillary

thyroid carcinomas (TPC1 and BCPAP), and an ATC (8505C). The identity of each of the cell lines was established by DNA fingerprinting as shown previously (25). Profiles were identical to the patterns published recently by Schweppe *et al.* (13). The mutational status of each of the thyroid cell lines has been verified. Cell lines were cultured at 37°C in air with 5% CO₂ under the conditions described previously (25). The nonthyroid human cell lines used were HeLa (cervix adenocarcinoma), human embryonic kidney (HEK), MCF7 (breast adenocarcinoma), and T98G (human glioma). HeLa, HEK, and T98G were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Life Technologies), and MCF7 was cultured in 1:1 RPMI 1640 containing L-glutamine and DMEM. Cells were cultured in the presence of 10% fetal calf serum, 1% sodium pyruvate, 2% streptomycin and penicillin, and 1% fungizone.

Cells were plated, and the next day, different doses of 5-AzadC (Sigma-Aldrich) ranging from 0.5, 1, and 5 μ M to 10 μ M were added for 3 to 7 days. Every 24 hours, the medium was replaced with fresh 5-AzadC, derived from a 10 mM stock solution dissolved in dimethylsulfoxide (DMSO). Cells were treated with HDAC inhibitors using concentrations of 50 nM, 100 nM, 500 nM, or 1 μ M of TSA (Sigma-Aldrich) derived from a 3.3 mM stock solution dissolved in ethanol, or with 1 μ M SAHA (Sigma-Aldrich) derived from a 50 mM stock solution that was prepared with DMSO. Cells were treated with an HDAC inhibitor alone ranging from 24 to 72 hours or in combination with 5-AzadC. Forskolin (AG Scientific, Inc.), dissolved in ethanol, was used at 10 μ M during 24 hours. All stock solutions were stored at –20°C. Total RNA was isolated using TRIzol Reagent, followed by a purification on RNeasy columns (Qiagen). RNA was used for semi- or quantitative RT-PCR and microarray analysis after verification of its quality as described previously (27).

RT-PCR

The effect of drug treatment on the mRNA expression of thyroid-specific markers, including *TSHR*, thyroperoxidase (*TPO*), thyroglobulin (*Tg*), *NIS*, dual oxidase 1 and 2 (*DUOX1* and *DUOX2*), and paired box gene 8 (*PAX8*) was investigated by RT-PCR as described previously (25), but using 35 cycles. mRNA expression patterns were compared to the expression of porphobilinogen deaminase (*PBGD*) (25). The expression of these genes was also investigated using another protocol in which PCRs contained 0.1 μ g cDNA, 1 $\times$ PCR Buffer (containing 15 mM MgCl₂; Qiagen), 5% DMSO, 0.2 mM dNTPs, 0.2 μ M of each primer, 1 $\times$ Q-Solution (Qiagen), and 0.67 μ L homemade *Taq* polymerase in a total volume of 50 μ L. PCR amplifications were performed as described previously (25), but using 35 cycles. All samples were analyzed on a 1% agarose gel and visualized with ethidium bromide.

Quantitative RT-PCR

NIS expression was investigated by quantitative RT-PCR using the forward primer 5'-TGC TCT TCA TGC CCG TCT TC-3' and the reverse primer 5'-AGC GCA TCT CCA GGT ACT CGT-3' under the conditions described by Burniat *et al.* (28) and using the *NIS* primers described by Hou *et al.* (23). *NIS* mRNA expression was normalized using neural precursor cell expressed developmentally downregulated 8 (*NEDD8*) and tetratricopeptide-repeat domain 1 (*TTC1*) as described previously (27).

Microarray analyses

The effect of 5-AzadC treatment on overall gene expression profiles was investigated using homemade spotted slides containing 25,344 spots as described previously (25,27). The microarray platform (GPL13666) has been submitted to Gene Expression Omnibus. Each experiment was performed in duplicate with swapped dyes. Data analyses were performed using BRB-ArrayTools developed by Dr. Richard Simon and the BRB-ArrayTools Development Team (29). Data were imported using the GenePix data importer, and ratios were flipped for reverse-fluor experiments, and background adjustment was applied. Both red and green spots with intensities below the minimum and flagged spots were excluded from the analysis. Data were normalized using lowess smoother (locally weighted scatterplot smoothing). Regulated genes were selected using significant analysis of microarrays (SAM) (30) as described previously (31), and data were visualized using the program R version 2.11.1 (32) and gplots package for R, version 2.8.0. Gene expression profiles of 5-AzadC-treated cell lines were compared to expression patterns of primary cultured thyrocytes treated with TSH (27,33) and untreated thyroid cell lines (25,33).

Furthermore, the strongest gene modulations induced by 5-AzadC in the cell lines were compared to their expression levels in thyroid cancers *in vivo*. These consisted of 11 ATC and a pool of normal thyroid tissues that had been hybridized on Affymetrix U133 Plus 2.0 arrays as described in Hébrant *et al.* (34). CEL files derived from ATCs and the pool of normal thyroid were normalized with GCRMA in GenePattern (35), and the expression of each gene was represented as the ratio between an ATC and the pool of normal thyroid tissues. 5-AzadC-regulated pathways were identified using the program DAVID Bioinformatics Resources (36).

To test for specific enrichment of our set of regulated genes (selected by SAM analysis), Gene Set Enrichment Analysis (GSEA; MsigDB) (37) was used in pairwise comparisons across the different phenotypes.

DNA quantification

A growth curve for each cell line was made starting from cell lines seeded at 2×10^5 cells. Cells were arrested each 24 hours until day 10. To investigate the effect of 5-AzadC on cell growth, cells were seeded at 50% of confluence in 10-cm Petri dishes. After 24 hours, cells were treated with 10 μ M 5-AzadC for 5 days, and the medium was replaced each day with fresh 5-AzadC. Control cells were treated similarly with or without 0.1% DMSO. Cells were arrested at consecutive days after 24 hours of 5-AzadC treatment until 5 days. They were detached during 10 minutes with $1 \times$ cold phosphate-buffered saline (PBS) containing 5 mM ethylene diamine tetraacetic acid (EDTA) and 5 mM ethylene glycol tetraacetic acid (EGTA) and collected by centrifugation at 560 g for 3 minutes. The pellet was rinsed with $1 \times$ PBS and digested overnight at 55°C in 600 μ L TNES buffer (100 mM Tris, 200 mM NaCl, 5 mM EDTA, and 0.2% SDS, pH 8) supplemented with 4 μ L proteinase K (Qiagen). Proteins were then precipitated with 200 μ L of 6 M NaCl and centrifuged at 13,000 g for 5 minutes at room temperature. DNA in the supernatant was precipitated with 600 μ L 100% isopropanol. The DNA pellet was washed with 600 μ L 70% ethanol and centrifuged at 13,000 g for 5 minutes at room temperature. The pellet was dried and re-

suspended overnight in 10 mM Tris containing 1 mM EDTA at room temperature. DNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer (Isogen Life-Science).

Viability assay

Cells were seeded at a density of 3×10^4 cells per well using 48-well plates. Twenty-four hours after plating, cells were treated with different concentrations of 5-AzadC (0.5, 1, and 5 μ M) or with 0.1% DMSO (controls), and the medium was changed with a fresh medium every day. Each condition was performed in triplicate for each cell line. Numbers of viable cells were determined after 1, 3, and 7 days of treatment using the CellTiter 96[®] Aqueous Nonradioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. This is a colorimetric method of cells based on the cleavage of the tetrazolium salt MTS by mitochondrial dehydrogenases in living cells. Briefly, the medium of each well was removed and replaced by 200 μ L of a solution containing 10 mL of culture medium, 2 mL MTS, and 100 μ L phenazine methosulfate. After 1 or 2 hours of incubation at 37°C, 100 μ L from each well was transferred into a 96-well plate, and the absorbance was measured at 490 nm using a plate reader (Microplate Reader Model 680; Bio-Rad). Viability was calculated as the ratio between the absorbance of 5-AzadC-treated cells and untreated cells and expressed as the percentage of control.

Statistical analysis

Software IBM-SPSS version 19 was used for the statistical analyses. For the DNA quantification, analysis of variance (ANOVA) for one repeated factor (time) at five levels, and one group factor (treatment) at three levels, was performed. This was followed by multiple treatment comparisons using Sidak tests. For MTS analyses, an ANOVA for one repeated factor (time) at three levels, one fixed factor (treatment) at four levels, and one random condition factor (different experiments) was performed. Next, multiple treatment comparisons were performed using Sidak tests.

Results

Effect of drugs inducing epigenetic modifications on the modulation of canonical thyroid-specific markers

Previously, we showed that a panel of commonly used human thyroid cancer cell lines had lost the expression of many thyroid differentiation markers, including *TSHR*, *NIS*, *Tg*, and *TPO*, but not thyroid transcription factors such as *PAX8* (25). To investigate whether the expression of differentiation markers could be induced in these cell lines, FTC133, BCPAP, TPC1, and 8505C cells were treated with a low (1 μ M) or high (10 μ M) concentration of 5-AzadC for 72 hours. The expression of each marker was investigated by RT-PCR and compared to the expression in thyroid tissue. *DUOX1* was not detected in TPC1 and 8505C cells under control conditions, but was expressed after treatment with 1 and 10 μ M 5-AzadC for 72 hours (Fig. 1A). Furthermore, the expression of *DUOX1* was increased compared to control cells both at 1 and 10 μ M of 5-AzadC in BCPAP cells. No effect on *DUOX1* was observed in FTC133. The expression of *DUOX2*, already observed in untreated BCPAP and TPC1 cells, was increased

FIG. 1. Semi-quantitative RT-PCR of thyroid differentiation markers after treatment of human thyroid cancer cell lines with 5-AzadC. **(A)** *DUOX1* and *DUOX2* mRNA expression patterns in four human thyroid tumor cell lines (FTC133, BCPAP, TPC1, and 8505C) after treatment with 1 or 10 μ M 5-AzadC for 72 hours compared to untreated cells (control, C) using the PCR protocol as described previously (25) with 35 cycles. **(B)** *NIS* mRNA expression patterns in BCPAP and TPC1 cell lines in untreated cells (C) and after treatment with 1 or 10 μ M 5-AzadC for 72 hours following the protocol as described in the Materials and Methods section using 35 cycles. Expression levels were compared to the mRNA levels of the housekeeping gene *PBGD*. (+), positive control consisting of thyroid RNA extracted from a patient with Graves' disease; (–), negative control (without cDNA in the amplification mixture). **(C)** *NIS* mRNA expression in the human thyroid tumor cell line FTC133. FTC133 cells were treated with 0.5 or 1 μ M of 5-AzadC during 7 days (7d) in the absence or presence of 100 nM TSA during the last 24, 48, or 72 hours or with TSA alone at the same time points. The expression patterns were compared to untreated FTC133 cells (C), a positive control consisting of a human primary culture of thyrocytes (PC), and a negative control (–) consisting of the PCR mixture without cDNA. 5-AzadC, 5-aza-2'-deoxycytidine; *DUOX1*, dual oxidase 1; *DUOX2*, dual oxidase 2; *PBGD*, porphobilinogen deaminase; TSA, trichostatin A; RT-PCR, reverse transcriptase–polymerase chain reaction.

after 5-AzadC treatments at both concentrations. No effect on *DUOX2* expression was detected in FTC133 or in 8505C cells. In these conditions, mRNAs of *TSHR*, *NIS*, *Tg*, and *TPO* were not detected in any of these cell lines, and 5-AzadC did not induce their expression (not shown). The expression of

thyroid markers was also investigated in FTC133, BCPAP, and TPC1 cells using a slightly different protocol, but keeping similar cDNA quantities and number of PCR cycles (see the Materials and Methods section). A faint *NIS* mRNA expression was detected in these three cell lines after treatment with 1 to 10 μ M 5-AzadC during 72 hours (Fig. 1B and Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/thy). These results were reproduced in six independent FTC133 experiments and in four out of five experiments for BCPAP (Supplementary Fig. S1). Since 5-AzadC is incorporated into DNA during proliferation, we reasoned that the level of plating density might affect expression levels of thyroid-specific genes. However, treatment with 5-AzadC during 72 hours of cells plated at different densities, that is, reaching 50%–80% of confluency, did not influence the expression of thyroid-specific genes (not shown). In addition, the effect of the HDAC inhibitor TSA on the expression of thyroid-specific genes was investigated with two PCR protocols. Treatment of thyroid cancer cell lines with TSA in concentrations varying from 50 nM, 100 nM, 500 nM, and 1 μ M for 24–72 hours did not induce the expression of thyroid-specific markers (not shown).

Effect of drug combinations and various culture conditions on *NIS* mRNA expression

Since *NIS* plays a crucial role in the treatment of thyroid cancer, we further investigated whether lower concentrations of 5-AzadC and longer treatment times could contribute to higher *NIS* mRNA expression levels. As synergistic effects have been described using 5-AzadC in combination with HDAC inhibitors (38), the cell lines were also treated in combination with TSA. In FTC133 cells treated for 7 days with 0.5 or 1 μ M 5-AzadC alone, a faint *NIS* mRNA expression was observed, which was increased when 5-AzadC was combined with 100 nM TSA during the last 24–72 hours (Fig. 1C). FTC133 cells treated with TSA alone did not show an expression of *NIS* mRNA (Fig. 1C). A combined treatment of 1 μ M 5-AzadC during 72 hours with 1 μ M SAHA during the last 24 hours was not different from 5-AzadC alone (not shown).

The presence of serum in the culture medium has been reported to affect *NIS* expression (39). To investigate whether reduced serum concentrations would favor differentiation, serum concentrations were diminished from 10% to 1%, and FTC133, BCPAP, and TPC1 cells were treated for 4 days with 1 μ M 5-AzadC in the absence or presence of 100 nM TSA during the last 24 hours. Furthermore, this treatment was combined with 10 μ M forskolin during the last 24 hours, an adenylate cyclase activator that induces differentiation in normal thyrocytes and increases *NIS* mRNA expression (39). Semiquantitative PCR showed the strongest induction of *NIS* mRNA expression in FTC133 using a 5-AzadC treatment for 96 hours in 1% serum combined with TSA and forskolin during the last 24 hours (not shown). *NIS* mRNA expression levels using combined drug treatments were further analyzed by qRT-PCR and compared to the expression in primary thyroid cultures treated with or without 0.3 mU/mL TSH during 72 hours ($n=3$). A small induction of *NIS* mRNA was measured in thyroid cancer cell lines after cotreatment with 5-AzadC, TSA, and forskolin; however, these levels did not reach basal *NIS* mRNA levels in untreated primary thyrocytes and were much lower than those induced by TSH in primary cultures (Fig. 2).

FIG. 2. Quantitative RT-PCR analysis of *NIS* mRNA expression levels in human thyroid tumor cell lines and primary cultured thyrocytes. Thyroid cancer cell lines were treated with 5-AzadC (A) for 96 hours, the last 24 hours in the presence of TSA (T) and forskolin (F) in the culture medium containing 1% serum. These were compared to cells in the same medium without the drug combination (C). Quantitative RT-PCR was performed with two different primer sets: derived from Hou *et al.* (23) (black bars), and with sequences described in the Materials and Methods section (white bars). All values were calculated using untreated primary cultures as a baseline set at 1.

Effect of 5-AzadC on overall gene expression: comparison with differentiated thyrocytes in vitro, nonthyroid cell lines, and dedifferentiated thyroid cancer cells in vivo

Previously, we studied microarray expression profiles of differentiated thyrocytes, that is, human thyrocytes in primary culture treated with TSH for different times (27). To investigate whether 5-AzadC might induce the expression of those differentiation-induced genes, the thyroid tumor cell lines FTC133, BCPAP, TPC1, and 8505C were treated with 1 or 10 μ M 5-AzadC during 72 hours, and their gene expression profiles were compared to those of differentiated thyrocytes *in vitro* (Fig. 3B). Figure 3B shows the expression patterns of commonly upregulated genes in primary thyrocytes treated with TSH for 16, 24, or 48 hours, selected by a one-class SAM, compared to their expression patterns in thyroid cell lines treated with 5-AzadC. Furthermore, these expression patterns were compared to profiles of nontreated thyroid tumor cell lines in relation to untreated cultured primary thyrocytes as shown previously (25). Although a number of genes had lower expression levels in cell lines compared to normal thyrocytes (Fig. 3B, *right*), 5-AzadC did not induce the overall expression of differentiation genes in the four different thyroid cell lines (Fig. 3B, *left*). In line with the PCR results of the canonical thyroid differentiation genes, although some modulations could be observed after 5-AzadC treatment, this compound did not strongly induce differentiation in the treated thyroid cancer cell lines. In addition, the genes regu-

lated in primary thyrocytes treated with TSH for 16–48 hours were used as a gene set in a GSEA analysis. This gene set was significantly enriched in short-time TSH-treated primary cultures compared to 5-AzadC-treated thyroid cell lines ($p=0.002$, normalized enrichment score [NES]=1.9). To further investigate which genes were induced by the 5-AzadC treatment, a one-class SAM analysis was used to identify the commonly modulated genes. These were included when q -values were <0.05 and with absolute values of $\log_2$ of expression ratios ≥ 0.58 , that is, fold-changes ≥ 1.5 . Among the genes identified by SAM, most were upregulated by the 5-AzadC treatment (Fig. 3A). Pathway analysis of commonly regulated genes by 5-AzadC in the cell lines, that is, genes depicted in Figure 3A, using DAVID software showed that modulated genes were involved in processes such as apoptosis, antigen presentation, defense response, cell migration, and response to extracellular signals (Supplementary Table S1). Comparison of 5-AzadC-modulated genes in cell lines with differentiated primary cultured thyrocytes showed again only few genes that were also modulated in differentiated thyrocytes *in vitro* (Fig. 3A). Indeed, GSEA analysis showed that the set of genes, commonly upregulated in 5-AzadC-treated thyroid cell lines, was not significantly enriched in the TSH-treated primary cultures ($p>0.25$).

Furthermore, we investigated whether 5-AzadC-induced genes were specific for thyroid cell lines or could also be observed in cell lines from nonthyroidal origin. Therefore, the expression of genes commonly regulated by 5-AzadC in thyroid cell lines (Fig. 3A) was compared to their level of expression in four human cell lines derived from different origins, that is, T98G (glioblastoma cell line), MCF7 (breast cancer cell line), HEK (human embryonic kidney cells), and HeLa (cervical cancer cell line) after treatment with 10 μ M 5-AzadC during 72 hours. Figure 3A shows an overlap in gene modulation by the demethylating drug in both thyroid and nonthyroid cell lines. Notably, 58 genes out of 145 (40%) were also regulated with an average fold-change of 1.5 or more in nonthyroid cell lines among which three genes, that is, *NNAT*, *ACTG1*, and *APC*, were modulated in all four nonthyroid cell lines. Taken as a gene set in a GSEA analysis, the 5-AzadC-modulated genes in thyroid cell lines were significantly more enriched in the nonthyroid cell lines treated with 5-AzadC than in the TSH-treated primary cultures ($p=0.002$, NES=1.9). Thus, cancer cell lines derived from different origins have relatively similar epigenetic modification patterns resulting in gene repression in continuous cultures.

To investigate whether the genes induced by 5-AzadC might play a role in thyroid tumorigenesis *in vivo*, the expression of the strongest commonly upregulated genes (average level of expression of twofold or more) by 5-AzadC treatment was compared to their expression levels in dedifferentiated thyroid cancer, that is, ATC (Fig. 3C). It was reasoned that if those genes were downregulated in ATC, this might have a potential therapeutic application. Figure 3C shows that from the 16 genes strongly induced by 5-AzadC, 12 were upregulated or nonregulated in most ATC samples compared to normal thyroid tissues.

Effect of 5-AzadC on cell growth and viability

It has been previously reported that 5-AzadC can induce not only differentiation but also cytotoxicity (24). Therefore,

FIG. 3. Heatmap of microarray data. Depicted genes were based on long-term TSH-induced genes in primary thyrocytes (**B**) or on 5-AzadC-induced genes in thyroid cancer cell lines (**A, C**). Upregulated genes were selected from a one-class SAM analysis (q -value < 0.05) on primary thyrocytes treated with 0.3 mU/mL TSH for 16, 24, or 48 hours (**B**) or a SAM analysis of 10 μ M 5-AzadC-treated cell lines (**A**), that is, FTC133 ($n=4$), BCPAP ($n=4$), TPC1 ($n=2$), and 8505C ($n=1$). SAM-selected genes had average values of expression $\geq 0.58 \log_2$ or $\leq -0.58 \log_2$, that is, 1.5-fold or more differentially expressed compared to untreated cells (**A, B**) or with an average fold change of twofold or more (**C**). In (**A**), expression of the SAM-selected genes was compared to their levels of expression in four human non-thyroid cell lines (T98G, MCF7, HEK, and HeLa) treated with 5-AzadC, and primary cultured thyrocytes treated with TSH for different times. In (**B**), expression of the SAM-selected genes was compared to their expression patterns in 5-AzadC-treated thyroid cell lines, short-term TSH-treated cultures, and untreated thyroid cell lines compared to normal thyrocytes in culture. In (**C**), SAM-selected genes were compared to their expression patterns in ATC that had been compared to a pool of normal thyroid tissues. Color scale bar indicates levels of differential gene expression, ranging from red to green representing high and low levels of modulations, respectively. Genes with fold changes > 3 ($\log_2$) were scaled to 3. Genes that were regulated < 1.5 -fold (between -0.58 and 0.58 in $\log_2$) were defined as nonregulated and are indicated in black. Missing values are indicated in gray. Data on human TSH-treated primary thyroid cell cultures and thyroid cell lines compared to normal thyrocytes were previously obtained (25,27). ATC, anaplastic thyroid carcinomas; SAM, significant analysis of microarrays; TSH, thyrotropin. (*continued*)

the effect of 5-AzadC on cell growth was investigated in human thyroid cancer cell lines. Comparison of the growth curves of untreated thyroid cell lines showed that FTC133 and BCPAP grew the fastest and the slowest, respectively (Fig. 4E). Treatment with 10 μ M 5-AzadC markedly reduced the DNA content in FTC133, BCPAP, TPC1, and WRO compared to untreated cells (Fig. 4). The means of the data calculated on

all treatment values, but separately for each time, were significantly different according to time ($p < 0.001$ for each of the four cell lines). Similarly, the means of the data calculated on all time values, but separately for each treatment, were significantly different according to time ($p < 0.001$ for each of the four cell lines). Moreover, the time evolution pattern was treatment dependent ($p < 0.001$; Supplementary Table S2). The effect of

FIG. 3. (continued)

reduced concentrations of 5-AzadC was then investigated combined with prolonged treatment times. The effect on cell viability was investigated after 1, 3, and 7 days of 5-AzadC treatment at concentrations of 0.5, 1, and 5 μ M in different thyroid cancer cell lines (Fig. 5). Overall, the type and time of treatment were found to have an effect ($p < 0.001$) for all the cell lines investigated (Supplementary Table S3). 5-AzadC reduced cell viability in each cell line in a concentration-dependent manner at 3 days of treatment, which was further decreased after 7 days of treatment (Fig. 5). WRO cells were the most affected by the treatment with 5-AzadC. When considering the overall effect of 5-AzadC treatment on viability, for all the cell

lines, 0.5, 1, and 5 μ M 5-AzadC-treated cells were significantly different from DMSO-treated controls, with p -values ranging from <0.001 to 0.008, except for one condition in 8505C cells (Supplementary Table S3).

Discussion

Loss of differentiation is a common event in tumor progression. For thyroid cancers, this often involves a loss of thyroid-specific functions, including a reduced expression or the complete lack of functional expression of NIS with loss of the possibility to accumulate RAI (40). These cancers

FIG. 4. DNA quantification of human thyroid cancer cell lines. (A–D) DNA was quantified from cell lines at consecutive days during 5 days from cells that were cultured in the medium alone (C) or supplemented with 0.1% DMSO (DMSO) or with 10 μ M 5-Aza-dC (5-Aza-dC). (E) DNA quantification of thyroid cell lines that were arrested at consecutive days during 10 days. Each condition was measured in duplicate for each time point and for each cell line. DMSO, dimethylsulfoxide.

therefore no longer respond to standard treatment with RAI therapy. Epigenetic silencing of several genes in thyroid tumors has been reported (41), such as the *TSHR* (7–9) and *NIS* (10,11). Based on these observations, a re-expression of these hypermethylated genes might be expected using demethylating agents such as 5-Aza-dC. Indeed, a number of studies have investigated the effect of epigenetic drug treatments on differentiation expression in cancer cell lines, whether or not from thyroidal origin. In some cell lines, re-expression of differentiation makers has been reported, but it was absent in others (14–23). To explore this further, we investigated systematically which genes could be reinduced in a panel of human thyroid tumor cell lines that have lost the expression of most classical thyroid differentiation genes (25) by using chromatin-modifying drugs in different conditions, that is, varying concentrations, treatment times, drug combinations, and culture conditions.

From the panel of thyroid-specific genes investigated by RT-PCR, the strongest effect was observed on *DUOX* expression after 5-Aza-dC treatment. A re-expression of *DUOX* after 5-Aza-dC treatment has also been reported recently using human lung cell lines (42). We observed that *NIS* mRNA expression could be slightly induced by 5-Aza-dC in human thyroid tumor cell lines from various origins and harboring different mutations. However, neither *NIS* protein nor significant iodide uptake could be detected (not shown). This might be explained by the results obtained by qRT-PCR, which showed that the levels of *NIS* mRNA expression were low compared to the expression in primary cultured thyrocytes. Therefore, this might not be sufficient to detect *NIS* protein expression nor lead to functional effects, that is, uptake of iodide. Other additional factors might be required that could affect the expression of *NIS*. Culture conditions can influence *NIS* functionality as shown for primary cultured thyrocytes in a

FIG. 5. Cell viability measurements in human thyroid cancer cell lines. Thyroid cancer cell lines were treated with 0.5, 1, or 5 μ M of 5-AzadC or 0.1% DMSO (controls), and cell viability was measured after 1, 3, and 7 days of treatment. Values are expressed as ratios of the absorbance of treatments over controls for each concentration and time point. Measurements were performed in two [FTC133 (A), BCPAP (B), WRO (D), and 8505C (E)] or three [TPC1 (C)] independent experiments per cell line, and each condition was measured in triplicate. T, 5-AzadC treatment; C, control (untreated cells).

monolayer or in follicle structure (43,44), or in serum and can reduce the response to TSH and the ensuing iodide uptake (39,45). However, neither cultivation of these cell lines in three-dimensional structures (not shown), whether combined with 5-AzadC treatments or not, nor reduced serum concentrations increased *NIS* mRNA expression. Comparison of 5-AzadC-induced genes in the cell lines with gene expression profiles of TSH differentiated thyrocytes showed only a few commonly expressed genes. Therefore, taken together, our results on thyroid cancer cell lines suggest that 5-AzadC, whether or not combined with TSA and forskolin, had only a minor effect on the induction of differentiation genes.

To get further insights into whether the genes re-induced by 5-AzadC could have a role in thyroid tumorigenesis, we compared the strongest 5-AzadC-induced genes in thyroid cancer cell lines with their levels of expression in ATC. We reasoned that if those genes would be downregulated in ATC (e.g., tumor repressor genes), silencing of these genes may have contributed to tumorigenesis. Therefore, re-expression of those genes by a demethylating agent could offer a therapeutic application. However, most genes were already upregulated in ATC, although a few genes were indeed repressed,

suggesting a potential therapeutic application for the latter. Nevertheless, our results on thyroid cancer cell lines, namely, the low *NIS* mRNA induction, as well as the absence of crucial proteins needed for iodide organification such as TPO and Tg, do not suggest a potential utility of 5-AzadC to reinduce differentiation genes in dedifferentiated *in vivo* thyroid cancers. Moreover, even re-expression of *NIS* mRNA may not be sufficient to restore iodide transport in thyroid carcinomas, because the *NIS* protein is often not localized at the cell membrane (46–48). However, one caveat remains: negative results in thyroid cancer cell lines do not necessarily imply negative results in *in vivo* tumors.

Further analyses of which genes were induced by the 5-AzadC treatment showed that they were implicated in various processes, including apoptosis or regulation of proliferation, which is in line with the obtained results on reduced cell growth and cell viability. The modulation of genes can be due to the direct demethylation of their promoters, but also to indirect effects, for example, a demethylated gene that induces the transcription of another gene, or it could be a consequence from the (adaptive) response of the cells to the treatment. The nature of the 5-AzadC-induced genes, that is,

the epigenetically silenced genes in the cell lines, could suggest that these genes have been silenced during serial culturing, because they were not required for the growth of cells cultivated *in vitro*, for example, genes involved in immune response or cell migration. A part of the 5-AzadC-modulated genes were also induced by the same treatment of nonthyroid cell lines. Individual pathway analysis of each cell line treated with 5-AzadC showed that additional pathways for each cell line were regulated (not shown).

Interestingly, clinical trials investigating the use of HDAC inhibitors and demethylating agents in the treatment of thyroid cancer have been performed or are still ongoing, as reviewed recently by Russo *et al.* (49). In a study of Kelly *et al.*, six patients with metastatic thyroid cancer (four poorly differentiated papillary thyroid cancers [PTCs], one Hürthle cell, and one medullary thyroid cancer) were treated with SAHA (50). Three patients with PTC underwent post-therapy RAI scans, and one had an improved uptake after treatment with oral SAHA. Five had stable disease after treatment, and one patient had a partial response. In another study published by Woyach *et al.*, the effect of SAHA was investigated in a larger group of patients with thyroid cancer. The authors concluded that at the dose and schedule used, SAHA was not an effective treatment of patients with advanced thyroid cancer (51). In this study, the use of combinatorial drug treatment was suggested. To our knowledge, a clinical study with decitabine is still ongoing (52). The comparison of the clinical outcome of decitabine treatment with the results of 5-AzadC-treated thyroid cancer cell lines will provide more insights into the use of this model for drug testing.

We observed that DNA quantities and cell viability measurements were lower for 5-AzadC-treated cell lines compared to untreated cells. This suggests that 5-AzadC treatment reduced the growth and/or the number of cells. 5-AzadC has been shown to reduce the growth of thyroid cell lines (20). This can be due to an inhibition of cell growth, an induction of cell death, a loss of cell adhesion, or a combination of these different effects. Thus, the arrest of cell growth does not induce a redifferentiation process. By microscopic evaluation of the cell lines during 5-AzadC treatment, we only observed a strong effect in WRO cells, reflected by a large number of floating cells. The viability assay confirmed that WRO cells were the most sensitive to 5-AzadC treatment. If such results could be extrapolated to *in vivo* tumors, epigenetic treatment might at least slow the growth of the tumor. However, it could also have deleterious effects such as in the generation of tumors in the hyperplastic thyroids of mice treated with 5-AzadC or 5-azacytidine (53).

One might envisage two categories of reinducible genes with low basal expression levels in the cell lines. A first group of genes, probably already silenced *in vivo*, includes genes implicated in differentiation that were either not or only slightly reinduced, although other tissue-specific determination genes such as the transcription factors *NKX2-1*, *FOXE1*, and *PAX8* were previously observed to be expressed in thyroid cancer cell lines (25). A second group of genes includes genes coding for proteins whose role became unnecessary in the newly acquired *in vitro* environment and those can be hypothesized to be epigenetically repressed as an adaptive response to these new conditions. This would be the case of genes participating in antigen presentation,

defense response, cell migration, or in response to extracellular signals. A part of those genes were also modulated by 5-AzadC in nonthyroid cell lines, which would support this hypothesis.

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References

1. Kondo T, Ezzat S, Asa SL 2006 Pathogenetic mechanisms in thyroid follicular-cell neoplasia. *Nat Rev Cancer* **6**: 292–306.
2. 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.
3. Brabant G, Maenhaut C, Kohrle J, Scheumann G, Dralle H, Hoang-Vu C, Hesch RD, von zur MA, Vassart G, Dumont JE 1991 Human thyrotropin receptor gene: expression in thyroid tumors and correlation to markers of thyroid differentiation and dedifferentiation. *Mol Cell Endocrinol* **82**: R7–R12.
4. Nikiforov YE, Nikiforova MN 2011 Molecular genetics and diagnosis of thyroid cancer. *Nat Rev Endocrinol* **7**:569–580.
5. Catalano MG, Poli R, Pugliese M, Fortunati N, Boccuzzi G 2010 Emerging molecular therapies of advanced thyroid cancer. *Mol Aspects Med* **31**:215–226.
6. Bizhanova A, Kopp P 2009 Minireview: the sodium-iodide symporter NIS and pendrin in iodide homeostasis of the thyroid. *Endocrinology* **150**:1084–1090.
7. Hoque MO, Rosenbaum E, Westra WH, Xing M, Ladenson P, Zeiger MA, Sidransky D, Umbricht CB 2005 Quantitative assessment of promoter methylation profiles in thyroid neoplasms. *J Clin Endocrinol Metab* **90**:4011–4018.
8. Schagdarsurengin U, Gimm O, Dralle H, Hoang-Vu C, Dammann R 2006 CpG island methylation of tumor-related promoters occurs preferentially in undifferentiated carcinoma. *Thyroid* **16**:633–642.
9. Xing M, Usadel H, Cohen Y, Tokumaru Y, Guo Z, Westra WB, Tong BC, Tallini G, Udelsman R, Califano JA, Ladenson PW, Sidransky D 2003 Methylation of the thyroid-stimulating hormone receptor gene in epithelial thyroid tumors: a marker of malignancy and a cause of gene silencing. *Cancer Res* **63**:2316–2321.
10. Smith JA, Fan CY, Zou C, Bodenner D, Kokoska MS 2007 Methylation status of genes in papillary thyroid carcinoma. *Arch Otolaryngol Head Neck Surg* **133**:1006–1011.

11. Neumann S, Schuchardt K, Reske A, Reske A, Emmrich P, Paschke R 2004 Lack of correlation for sodium iodide symporter mRNA and protein expression and analysis of sodium iodide symporter promoter methylation in benign cold thyroid nodules. *Thyroid* **14**:99–111.
12. Venkataraman GM, Yatin M, Marcinek R, Ain KB 1999 Restoration of iodide uptake in dedifferentiated thyroid carcinoma: relationship to human Na⁺/I[−]-symporter gene methylation status. *J Clin Endocrinol Metab* **84**:2449–2457.
13. Schweppe RE, Kloppe JP, Korch C, Pugazhenth U, Benezra M, Knauf JA, Fagin JA, Marlow LA, Copland JA, Smallridge RC, Haugen BR 2008 Deoxyribonucleic acid profiling analysis of 40 human thyroid cancer cell lines reveals cross-contamination resulting in cell line redundancy and misidentification. *J Clin Endocrinol Metab* **93**:4331–4341.
14. Kitazono M, Robey R, Zhan Z, Sarlis NJ, Skarulis MC, Aikou T, Bates S, Fojo T 2001 Low concentrations of the histone deacetylase inhibitor, depsipeptide (FR901228), increase expression of the Na⁺/I[−] symporter and iodine accumulation in poorly differentiated thyroid carcinoma cells. *J Clin Endocrinol Metab* **86**:3430–3435.
15. Fortunati N, Catalano MG, Arena K, Brignardello E, Piovesan A, Boccuzzi G 2004 Valproic acid induces the expression of the Na⁺/I[−] symporter and iodine uptake in poorly differentiated thyroid cancer cells. *J Clin Endocrinol Metab* **89**:1006–1009.
16. Zarnegar R, Brunaud L, Kanauchi H, Wong M, Fung M, Ginzinger D, Duh QY, Clark OH 2002 Increasing the effectiveness of radioactive iodine therapy in the treatment of thyroid cancer using trichostatin A, a histone deacetylase inhibitor. *Surgery* **132**:984–990.
17. Furuya F, Shimura H, Suzuki H, Taki K, Ohta K, Haraguchi K, Onaya T, Endo T, Kobayashi T 2004 Histone deacetylase inhibitors restore radioiodide uptake and retention in poorly differentiated and anaplastic thyroid cancer cells by expression of the sodium/iodide symporter thyroperoxidase and thyroglobulin. *Endocrinology* **145**:2865–2875.
18. Provenzano MJ, Fitzgerald MP, Krager K, Domann FE 2007 Increased iodine uptake in thyroid carcinoma after treatment with sodium butyrate and decitabine (5-Aza-dC). *Otolaryngol Head Neck Surg* **137**:722–728.
19. Akagi T, Luong QT, Gui D, Said J, Selektor J, Yung A, Bunce CM, Braunstein GD, Koeffler HP 2008 Induction of sodium iodide symporter gene and molecular characterisation of HNF3 beta/FoxA2, TTF-1 and C/EBP beta in thyroid carcinoma cells. *Br J Cancer* **99**:781–788.
20. Vivaldi A, Miasaki FY, Ciampi R, Agate L, Collecchi P, Capodanno A, Pinchera A, Elisei R 2009 Re-differentiation of thyroid carcinoma cell lines treated with 5-Aza-2'-deoxycytidine and retinoic acid. *Mol Cell Endocrinol* **307**:142–148.
21. Miasaki FY, Vivaldi A, Ciampi R, Agate L, Collecchi P, Capodanno A, Pinchera A, Elisei R 2008 Retinoic acid receptor beta2 re-expression and growth inhibition in thyroid carcinoma cell lines after 5-aza-2'-deoxycytidine treatment. *J Endocrinol Invest* **31**:724–730.
22. Frohlich E, Brossart P, Wahl R 2009 Induction of iodide uptake in transformed thyrocytes: a compound screening in cell lines. *Eur J Nucl Med Mol Imaging* **36**:780–790.
23. Hou P, Bojdani E, Xing M 2010 Induction of thyroid gene expression and radioiodine uptake in thyroid cancer cells by targeting major signaling pathways. *J Clin Endocrinol Metab* **95**:820–828.
24. Jabbour E, Issa JP, Garcia-Manero G, Kantarjian H 2008 Evolution of decitabine development: accomplishments, ongoing investigations, and future strategies. *Cancer* **112**:2341–2351.
25. van Staveren WCG, Weiss Solis D, Delys L, Duprez L, Andry G, Franc B, Thomas G, Libert F, Dumont JE, Detours V, Maenhaut C 2007 Human thyroid tumor cell lines derived from different tumor types present a common dedifferentiated phenotype. *Cancer Res* **67**:8113–8120.
26. van Staveren WCG, Solis DY, Hébrant A, Detours V, Dumont JE, Maenhaut C 2009 Human cancer cell lines: experimental models for cancer cells *in situ*? For cancer stem cells? *Biochim Biophys Acta* **1795**:92–103.
27. van Staveren WCG, Weiss Solis D, Delys L, Venet D, Cappello M, Andry G, Dumont JE, Libert F, Detours V, Maenhaut C 2006 Gene expression in human thyrocytes and autonomous adenomas reveals suppression of negative feedbacks in tumorigenesis. *Proc Natl Acad Sci USA* **103**:413–418.
28. Burniat A, Jin L, Detours V, Driessens N, Goffard JC, Santoro M, Rothstein J, Dumont JE, Miot F, Corvilain B 2008 Gene expression in RET/PTC3 and E7 transgenic mouse thyroids: RET/PTC3 but not E7 tumors are partial and transient models of human papillary thyroid cancers. *Endocrinology* **149**:5107–5117.
29. Simon R, Lam A, Li MC, Ngan M, Menenzes S, Zhao Y 2007 Analysis of gene expression data using BRB-array tools. *Cancer Inform* **3**:11–17.
30. Tusher VG, Tibshirani R, Chu G 2001 Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* **98**:5116–5121.
31. Hébrant A, van Staveren WCG, Delys L, Solis DW, Bogdanova T, Andry G, Roger P, Dumont JE, Libert F, Maenhaut C 2007 Long-term EGF/serum-treated human thyrocytes mimic papillary thyroid carcinomas with regard to gene expression. *Exp Cell Res* **313**:3276–3284.
32. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J 2004 Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* **5**:R80.
33. Detours V; Université libre de Bruxelles. IRIBHM Microarray Data. Available online at www.ulb.ac.be//medecine/iribhm/microarray/data (accessed August 13, 2012).
34. Hébrant A, Dom G, Dewaele M, Andry G, Trésallet C, Leteurtre E, Dumont JE, Maenhaut C 2012 mRNA expression in papillary and anaplastic thyroid carcinoma: molecular anatomy of a killing switch. *PLoS One* **7**:e37807.
35. Reich M, Liefeld T, Gould J, Lerner J, Tamayo P, Mesirov JP 2006 GenePattern 2.0. *Nat Genet* **38**:500–501.
36. Huang dW, Sherman BT, Lempicki RA 2009 Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* **4**:44–57.
37. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP 2005 Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA* **102**:15545–15550.
38. Cecconi D, Donadelli M, Dalla PE, Rinalducci S, Zolla L, Scupoli MT, Righetti PG, Scarpa A, Palmieri M 2009 Synergistic effect of trichostatin A and 5-aza-2'-deoxycytidine on growth inhibition of pancreatic endocrine tumour cell lines: a proteomic study. *Proteomics* **9**:1952–1966.

39. Saito T, Endo T, Kawaguchi A, Ikeda M, Nakazato M, Kogai T, Onaya T 1997 Increased expression of the Na⁺/I⁻ symporter in cultured human thyroid cells exposed to thyrotropin and in Graves' thyroid tissue. *J Clin Endocrinol Metab* **82**:3331–3336.
40. Dohan O, De I, V, Paroder V, Riedel C, Artani M, Reed M, Ginter CS, Carrasco N 2003 The sodium/iodide symporter (NIS): characterization, regulation, and medical significance. *Endocr Rev* **24**:48–77.
41. Huang Y, de la CA, Pellegata NS 2003 Hypermethylation, but not LOH, is associated with the low expression of MT1G and CRABP1 in papillary thyroid carcinoma. *Int J Cancer* **104**:735–744.
42. Luxen S, Belinsky SA, Knaus UG 2008 Silencing of DUOX NADPH oxidases by promoter hypermethylation in lung cancer. *Cancer Res* **68**:1037–1045.
43. Bernier-Valentin F, Trouttet-Masson S, Rabilloud R, Selmi-Ruby S, Rousset B 2006 Three-dimensional organization of thyroid cells into follicle structures is a pivotal factor in the control of sodium/iodide symporter expression. *Endocrinology* **147**:2035–2042.
44. Kogai T, Curcio F, Hyman S, Cornford EM, Brent GA, Hershman JM 2000 Induction of follicle formation in long-term cultured normal human thyroid cells treated with thyrotropin stimulates iodide uptake but not sodium/iodide symporter messenger RNA and protein expression. *J Endocrinol* **167**:125–135.
45. Kraiem Z, Sadeh O, Heinrich R 2000 Preparation and culture of a serum-free human thyroid follicle system and its application for measuring thyroid hormone secretion, iodide uptake and organification, cyclic adenosine monophosphate formation, gene expression, and cell growth. *Thyroid* **10**:53–57.
46. Wapnir IL, van de RM, Nowels K, Amenta PS, Walton K, Montgomery K, Greco RS, Dohan O, Carrasco N 2003 Immunohistochemical profile of the sodium/iodide symporter in thyroid, breast, and other carcinomas using high density tissue microarrays and conventional sections. *J Clin Endocrinol Metab* **88**:1880–1888.
47. Peyrottes I, Navarro V, Ondo-Mendez A, Marcellin D, Bellanger L, Marsault R, Lindenthal S, Ettore F, Darcourt J, Pourcher T 2009 Immunoanalysis indicates that the sodium iodide symporter is not overexpressed in intracellular compartments in thyroid and breast cancers. *Eur J Endocrinol* **160**:215–225.
48. Kollecker I, von WR, Langner C, Mueller JA, Spitzweg C, Kreipe H, Brabant G 2012 Subcellular distribution of the sodium-iodide symporter (NIS) in benign and malignant thyroid tissues. *Thyroid* **22**:529–535.
49. Russo D, Damante G, Puxeddu E, Durante C, Filetti S 2011 Epigenetics of thyroid cancer and novel therapeutic targets. *J Mol Endocrinol* **46**:R73–R81.
50. Kelly WK, O'Connor OA, Krug LM, Chiao JH, Heaney M, Curley T, MacGregore-Cortelli B, Tong W, Secrist JP, Schwartz L, Richardson S, Chu E, Olgac S, Marks PA, Scher H, Richon VM 2005 Phase I study of an oral histone deacetylase inhibitor, suberoylanilide hydroxamic acid, in patients with advanced cancer. *J Clin Oncol* **23**:3923–3931.
51. Woyach JA, Kloos RT, Ringel MD, Arbogast D, Collamore M, Zwiebel JA, Grever M, Villalona-Calero M, Shah MH 2009 Lack of therapeutic effect of the histone deacetylase inhibitor vorinostat in patients with metastatic radioiodine-refractory thyroid carcinoma. *J Clin Endocrinol Metab* **94**:164–170.
52. Sherman S; National Cancer Institute. Decitabine in treating patients with metastatic papillary thyroid cancer or follicular thyroid cancer unresponsive to iodine I 131. Available online at <http://clinicaltrials.gov/ct2/show/NCT00085293> (accessed August 13, 2012).
53. Thomas GA, Williams ED 1992 Production of thyroid tumours in mice by demethylating agents. *Carcinogenesis* **13**:1039–1042.

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