Different expression of TSH receptor and *NIS* genes in thyroid cancer: role of epigenetics

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Abstract

The TSH receptor (TSHR) and sodium/iodide symporter (NIS) are key players in radioiodine-based treatment of differentiated thyroid cancers. While *NIS* (*SLC5AS*) expression is diminished/lost in most thyroid tumors, *TSHR* is usually preserved. To examine the mechanisms that regulate the expression of *NIS* and *TSHR* genes in thyroid tumor cells, we analyzed their expression after inhibition of ras–BRAF–MAPK and PI3K–Akt–mTOR pathways and the epigenetic control occurring at the gene promoter level in four human thyroid cancer cell lines. Quantitative real-time PCR was used to measure *NIS* and *TSHR* mRNA in thyroid cancer cell lines (TPC-1, BCPAP, WRO, and FTC-133). Western blotting was used to assess the levels of total and phosphorylated ERK and Akt. Chromatin immunoprecipitation was performed for investigating histone post-translational modifications of the *TSHR* and *NIS* genes. ERK and Akt inhibitors elicited different responses of the cells in terms of *TSHR* and *NIS* mRNA levels. Akt inhibition increased *NIS* transcript levels and reduced those of *TSHR* in FTC-133 cells but had no significant effects in BCPAP. ERK inhibition increased the expression of both genes in BCPAP cells but had no effects in FTC-133. Histone post-translational modifications observed in the basal state of the four cell lines as well as in BCPAP treated with ERK inhibitor and FTC-133 treated with Akt inhibitor show cell- and gene-specific differences. In conclusion, our data indicate that in thyroid cancer cells the expression of *TSHR* and *NIS* genes is differently controlled by multiple mechanisms, including epigenetic events elicited by major signaling pathways involved in thyroid tumorigenesis.

Key Words

- NIS
- TSH receptor
- thyroid cancer
- MAPK
- Akt
- epigenetics

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Introduction

Loss of differentiation is a common feature of thyroid cancers, and it has important prognostic implications. Radioactive iodine is the most effective method for managing recurrent and metastatic disease in these

patients, but it requires adequate tumor tissue expression of genes that play key roles in iodine metabolism, in particular those encoding the thyroid-stimulating hormone receptor (TSHR) and the sodium/iodide

symporter (NIS) (Schlumberger *et al.* 2007, Wartofsky & Van Nostrand 2012). Many studies have demonstrated losses/reductions in NIS (*SLC5AS*) expression and function in primary and metastatic thyroid cancer cells as a result of oncogenic activation (Trapasso *et al.* 1999, Arturi *et al.* 2000, Puxeddu *et al.* 2008, Kogai & Brent 2012). Alterations involving the ras–RAF–MAPK and PI3K–Akt–mTOR signal transduction pathways have been shown to play major roles in these changes (Xing 2010). Important contributions are also made by epigenetic events, such as changes in the methylation and acetylation of histones at the level of gene promoters (Russo *et al.* 2011, Galvão *et al.* 2013). For this reason, histone deacetylase inhibitors are being investigated as a potential means for restoring NIS expression and function in thyroid cancer cells (Puppini *et al.* 2005, Russo *et al.* 2013). As far as *TSHR* is concerned, early studies found that the expression of this gene in most thyroid cancers is similar to or slightly lower than that found in normal thyrocytes (Brabant *et al.* 1991, Ohta *et al.* 1991, Lazar *et al.* 1999, Sheils & Sweenwy 1999, Tanaka *et al.* 2000, Gérard *et al.* 2003, Durante *et al.* 2007). Few data are available on the molecular mechanisms underlying the regulation of *TSHR* expression in thyroid cancer cells. Indeed, at variance with the reports in tumor tissues, loss of *TSHR* expression has been described in most of human thyroid cancer cells (Pilli *et al.* 2009; Sponziello M, Durante C, Russo D & Filetti S, 2013, unpublished observations). However, hypermethylation of the *TSHR* promoter has been documented in a series of thyroid cancer tissues (Xing *et al.* 2003).

Different mechanisms thus seem to govern the expression of these fundamental markers of differentiation in thyroid cancers. Elucidation of these mechanisms might reveal important clues for the development of more effective therapeutic options for thyroid malignancies that are unresponsive to radioiodine. To this end, we examined the effects of specific MAPK and Akt pathway inhibitors on the expression of these two genes in thyroid cancer cell lines characterized by different basal levels of *NIS*/*TSHR* expression and the role played by post-translational histone modifications in the changes in *NIS* and *TSHR* expression in the cells treated with these inhibitors.

Subjects and methods

Cell cultures

We obtained human papillary thyroid carcinoma cells (TPC-1 and BCPAP) from Dr E Puxeddu of the University of Perugia. Follicular thyroid cancer cells were supplied by

Dr P E Goretzki of the Tumor Center Neuss-Düsseldorf (FTC-133 cells) and Dr A Fusco of the University of Naples (WRO cells). The thyroid origins of all cell lines were confirmed by genotype analysis (Schweppe *et al.* 2008). The cells cultured as described previously (Arturi *et al.* 2001), were treated with the MEK inhibitor U0126 (10 µM in DMSO) and Akt inhibitor IV (B2311; 0.5 µM in DMSO) (both from Sigma–Aldrich; Hou *et al.* 2009). Control cells were exposed to DMSO alone at the same concentration used in U0126- and B2311-treated cultures.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays

Cellular viability was evaluated with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Briefly, cells were seeded into 96-well plates (density, 3.5×10^3 cells/well for FTC-133 and 6×10^3 cells/well for BCPAP). The next day, the medium was replaced with a fresh medium, DMSO alone (controls) or containing U0126 (10 µM) or B2311 (0.5 µM). After 24 and 48 h of incubation, the medium was replaced with phenol red-free medium containing MTT (0.5 mg/ml; Sigma–Aldrich). Four hours later, the solubilized formazan product was quantified with a microplate spectrophotometer (xMark Spectrophotometer Microplate, Bio-Rad) at a wavelength of 540–690 nm.

RNA extraction and real-time PCR

The TRIzol method (Invitrogen) was used to extract total RNA from thyroid cancer cells (untreated or treated with U0126 or B2311). *TSHR* and *NIS* levels were determined with real-time quantitative RT-PCR, as described previously (D'Agostino *et al.* 2012, Celano *et al.* 2013). The same reaction was performed for the evaluation of the transcript levels of thyroglobulin (*Tg*), thyroperoxidase (*TPO*), *PAX8*, *NKX2.1*, and *FOXE1* genes. Two micrograms of RNA were reverse transcribed in a 20 µl reaction volume using the High Capacity cDNA Kit (Applied Biosystems). The cDNAs were then diluted 1:5 in nuclease-free H₂O (Gibco) and then amplified in an Applied Biosystems 7900HT fast real-time PCR Sequence Detection System (Applied Biosystems) using fast quantitative PCR thermal cycler parameters. A 20 µl reaction contained 1 µl of cDNA, 10 µl TaqMan Fast Universal PCR Master Mix (Applied Biosystems), and 1 µl of a primer/probe mixture for each gene evaluated. The β-actin gene was used as an endogenous reference. All amplification reactions were performed in triplicate, and the threshold cycles

(identified with Applied Biosystems Software, SDS version 2.2.1) of the three reactions were averaged. Results were obtained by the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001) and normalized to a sample of control (untreated) cells.

Protein extraction and western blotting

Total proteins were extracted from the cells treated with U0126 or B2311, as described previously (D'Agostino *et al.* 2012). The cell lysates were quantified spectrophotometrically using the Bradford method. The samples (20 µg) were loaded onto a 12% SDS–polyacrylamide gel and subjected to electrophoresis at a constant voltage (120 V). The resolved proteins were transferred to Hybond-P ECL nitrocellulose membranes (GE Healthcare, Milan, Italy) and blocked for 1 h at room temperature with TTBS/milk (TBS, 1% Tween 20, 5% nonfat dry milk). The membranes were then incubated overnight at 4 °C with the following primary antibodies (in TTBS/milk): polyclonal anti-Akt (1:1000) and anti-phospho-Akt antibodies (1:500), both from Cell Signaling Technology (Danvers, MA, USA); polyclonal anti-ERK1,2 (1:2000) and monoclonal anti-phospho-ERK1,2 (1:1000) antibodies (Santa Cruz Biotech.). After one 15-min and two 5-min washes in TTBS, the membranes were incubated with HRP-conjugated secondary antibodies (Transduction Laboratories, Lexington, TX, USA; 1:10 000) in TTBS/milk, and the proteins were visualized with ECL Prime Reagent (GE Healthcare).

Global histone modifications were analyzed after histone acidic extraction. Briefly, the cell pellet was suspended in HCl 0.5 M added with phenylmethylsulphonyl fluoride and a Protease Inhibitor Cocktail (Sigma–Aldrich). Then the lysate was sonicated for 10 s three times and centrifuged at 2500 g, 10 min, 4 °C. The supernatant was collected and processed twice as described earlier. The total supernatant of the three extractions was precipitated with ten volumes of cold acetone, at –20 °C overnight. The sample was then centrifuged at 2500 g, 15 min, 4 °C; the supernatant was discarded and the pellet was resuspended in water. For western blot analysis, 15 µg protein extracts were electrophoresed on 18% SDS–PAGE. Proteins were then transferred to nitrocellulose membranes and were saturated by incubating for 1 h with 5% nonfat dry milk in PBS/0.1% Tween 20. The membranes were then incubated overnight with rabbit polyclonal anti-acetyl-histone H3 antibody (Millipore Corporation, Billerica, MA, USA), histone H3 trimethyl Lys4 antibody (Active Motif, Carlsbad, CA, USA), histone H3 trimethyl Lys27 antibody

(Active Motif), or rabbit polyclonal anti H3 antibody (BioVision, Inc. Headquarters, Milpitas, CA, USA). After three washes with PBS 0.1% Tween 20, the membranes were incubated with anti-rabbit immunoglobulin coupled with peroxidase (Sigma–Aldrich). After 2 h of incubation, the membranes were washed three times with PBS 0.1% Tween 20, and the blots were developed using Chemidoc XRS (Bio-Rad) with the chemiluminescence procedure (GE Healthcare).

Chromatin immunoprecipitation

Cross-linkage of control and test substance-treated TPC-1, WRO, BCPAP, and FTC-133 cells was produced by exposure to formaldehyde (final concentration, 1%) at 37 °C for 10 min before harvesting. The cells were then washed twice with ice-cold PBS, scraped into 1 ml of ice-cold PBS supplemented with protease inhibitors, and centrifuged for 5 min at 3500 g (4 °C). The cell pellets were suspended in the cell lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1, and protease inhibitors) and incubated on ice for 10 min. The lysed samples were sonicated to generate chromatin fragments with an average length of ~200–600 bp. After centrifugation at 11000 g for 10 min at 4 °C, the supernatants were collected, and the samples were diluted tenfold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, and 167 mM NaCl). An aliquot (10%) saved as 'total input chromatin' was processed with the eluted immunoprecipitates, beginning with the cross-link reversal step. The samples were incubated (30 min, 4 °C, on a rotating platform) for preclearing with 80 µl of salmon sperm DNA/protein-A–agarose beads. The beads were then centrifuged (1 min at 3500 g), and the supernatant fractions were collected and immunoprecipitated overnight at 4 °C with 10 µg of anti-H3 acetylated polyclonal rabbit antibody (cat no. 06-599, Upstate, Millipore Corporation, Lake Placid, NY, USA); H3K4 trimethyl polyclonal rabbit antibody (cat no. 39159, Active Motif); or H3K27 trimethyl polyclonal rabbit antibody (cat no. 39155, Active Motif). All of these antibodies are appropriate for chromatin immunoprecipitation (ChIP) assays, as demonstrated in previous studies (Braunstein *et al.* 1996, Ieda *et al.* 2010, Lavarone *et al.* 2013). The samples and negative controls were incubated with 60 µl of salmon sperm DNA/protein-A–agarose beads for 1 h at 4 °C. The beads were pelleted by centrifugation and washed for 5 min on a rotating platform with 1 ml of each of the following buffers: low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, and

Table 1 Primers used for readout of ChIP assays

	Primer forward	Primer reverse	Probe
<i>NIS</i>	TGCCCTCTCTG- AGCTCAAT	CCTGATCACAGCTGT- CACTGTCT	TCCCCACCTGT- CAAC
<i>TSHR</i>	CCACAGTGGT- GAGGTCACA	GTTACCCAGGC- CAAAGGA	CCTTGAGCC- CTCCCTC

150 mM NaCl); high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, and 500 mM NaCl); LiCl wash buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, and pH 8.1); and TE buffer (10 mM Tris-HCl, 1 mM EDTA, and pH 8.0). After each wash, samples were centrifuged at 4 °C for 1 min at 500 g and the supernatant was discarded. Immunocomplexes were eluted from beads with elution buffer (1% SDS and 0.1 M NaHCO₃), and cross-linkage was reversed by incubation at 65 °C for 4 h. After the addition of proteinase K, Tris-HCl pH 6.5, and EDTA 0.5 M, the samples were incubated for 1 h at 45 °C. DNA was purified with phenol/chloroform extraction followed by ethanol precipitation and used as a template in quantitative absolute PCR with the reaction primers listed in Table 1. After PCRs, acetylated or methylated H3 levels were measured and expressed as ratios of signals recorded after and before (input) immunoprecipitation.

Statistical analysis

TSHR and *NIS* mRNA levels were expressed as means $\pm$ s.d., and differences were analyzed with the Tukey-Kramer multiple comparisons test performed with GrafPAD Software for Science (San Diego, CA, USA). *FOXE1*, *NKX2.1*, *Tg*, and *PAX8* mRNA levels were expressed as means $\pm$ s.d., and the significance of differences was assessed with the *t*-test. *P* values <0.05 were regarded as statistically significant. Data analysis was performed by using StatView 5.0.1 Software (SAS Institute, Inc., Cary, NC, USA).

Results

TSHR and *NIS* gene expression and regulation in thyroid cancer cell lines

Our first goal was to identify the regulatory mechanisms responsible for the *NIS* and *TSHR* mRNA expression patterns described in thyroid tumors. *In vitro* experiments were conducted in four human thyroid cancer cell lines characterized by different *NIS* and *TSHR*

expression profiles. As shown in Fig. 1A, *TSHR* mRNA was detectable (i.e. present after fewer than 38 cycles of amplification) in BCPAP cells but not in TPC-1 cells (both of which are PTC-derived). The highest levels (appearance after 30 cycles of amplification) were observed in FTC-133 cells, and no *TSHR* transcript was detected in the second follicular cell line, WRO. In contrast, *NIS* mRNA was detected only in WRO and BCPAP cells (threshold cycles: 36.0 and 36.7 for WRO and BCPAP respectively, 37.8 for FTC-133 cells; Fig. 1A).

To determine whether these patterns reflect cell-line-specific forms of epigenetic control of gene expression, we performed ChIP experiments using primers specific for the minimal promoter sequences of *TSHR* and *NIS* (Fig. 1B). We evaluated the presence of histone H3 marks associated with active transcription (i.e. acetylation of lysines 9 and 14 (H3K9K14ac), trimethylation of lysine 4 (H3K4me3); Barsky *et al.* 1997, Santos-Rosa *et al.* 2002, Pokholok *et al.* 2005, Rando 2007) or with transcriptional repression (i.e. trimethylation of lysine 27 (H3K27me3); Barsky *et al.* 1997, Füllgrabe *et al.* 2011). In experiments focusing on the *TSHR* promoter, FTC-133 cells displayed the highest levels of H3K9K14ac and H3K4me3 (transcription-permissive) and the lowest levels of H3K27me3 (transcription-repressive), whereas BCPAP, TPC-1, and WRO cells all had very high levels of H3K27me3 (Fig. 1B). As for the *NIS* gene promoter, the post-translational H3 modifications we analyzed showed no correlation with *NIS* mRNA levels observed in the cell lines. Indeed, H3K27me3 levels were highest in WRO cells, which had exhibited the highest levels of *NIS* mRNA (Fig. 1B).

These data clearly indicate that *TSHR* and *NIS* transcript levels in FTC-133 cells are subjected to different forms of epigenetic control. In the context of current models of epigenetic regulation of gene expression (Barsky *et al.* 1997, Santos-Rosa *et al.* 2002, Pokholok *et al.* 2005, Rando 2007, Füllgrabe *et al.* 2011), the post-translational H3 modifications in the *TSHR* promoter region are fully consistent with the *TSHR* mRNA levels observed in the same cell lines, but this is not true for the *NIS* gene (Barsky *et al.* 1997, Santos-Rosa *et al.* 2002, Pokholok *et al.* 2005, Rando 2007, Füllgrabe *et al.* 2011).

Modulation of two major signal transduction pathways involved in thyroid tumorigenesis

In the next set of experiments, we exposed FTC-133 and BCPAP cells (the two lines with detectable levels of *TSHR* mRNA) to the MEK inhibitor U0126 (10 μ M) and the

Figure 1

Expression levels and histone post-translational modifications of *TSHR* and *NIS* genes in TPC-1, BCPAP, WRO, and FTC-133 cells. (A) mRNA levels. RNA extraction and real time PCR are described in 'Subjects and methods' section. For each cell line the results were normalized against β -actin and expressed in arbitrary unit ($2^{-\Delta Ct}$). (B) Histone H3 post-translational

modifications. Bars indicate levels of acetylation at lysines 9 and 14 (top), trimethylation at lysine 4 (middle), and trimethylation at lysine 27 (bottom). Each bar represents the mean value of three different determinations. For each bar s.d. is not above 15% of each value.

Akt inhibitor B2311 (0.5 μ M). Both have been shown to induce NIS expression and function in other models of thyroid and non-thyroid cancer cells (Kogai *et al.* 2008, Hou *et al.* 2010, Liu *et al.* 2012).

As shown in Fig. 2A, western blotting showed that, at the doses used, U0126 and B2311 clearly reduced the phosphorylation of ERK or Akt respectively in both cell lines. However, the effects of inhibitors on *NIS* and *TSHR* mRNA levels were both gene- and cell line-specific (Fig. 2B). After 30 h of exposure to B2311, FTC-133 cells exhibited upregulated *NIS* transcription (~ 2.5 -fold increases over control levels, $P < 0.01$) and downregulated expression of *TSHR* (approximately fivefold reductions compared with controls, $P < 0.01$), but no significant changes in the expression of either gene were detected in BCPAP cells. In contrast, MEK inhibition with U0126 significantly increased the expression of both *NIS* and *TSHR* in BCPAP cells but had no effects in FTC-133 cells (Fig. 2B). In addition to *NIS* and *TSHR*, effects of U0126 and B2311 on the expression of *PAX8*, *NKX2.1*, *FOXO1*, *Tg*, and *TPO* genes were evaluated in FTC-133 and BCPAP cells. Both in the basal and in stimulated states *TPO* was never

detectable in all these cell lines. As shown in Fig. 3, the general profile of thyroid-specific gene expression after ERK and Akt inhibition appears to be gene- and cell line-specific.

To determine whether post-translational modifications of histone H3 were involved in the changes in *TSHR* and *NIS* expression induced by the two inhibitors, we performed ChIP assays on BCPAP cells treated for 24 h with U0126 and on FTC-133 cells exposed for 24 h to B2311. The results are shown in Fig. 4A. In FTC-133 cells, B2311-mediated AKT inhibition reduced H3K9K14ac and H3K4me3 levels (transcription activation marks) and increased levels of H3K27me3 (transcription repression) at the level of the *TSHR* promoter, findings that were consistent with the *TSHR* transcript levels shown in Fig. 2B. In contrast, at the *NIS* gene, instead, the same treatment decreased H3K9K14ac and H3K4me3 levels and increased levels of H3K27me3. In BCPAP cells, where MEK inhibition had increased *NIS* and *TSHR* mRNA levels (Fig. 2B), U0126 increased transcriptional activation marks (H3K9K14ac and H3K4me3) at the *TSHR* promoter, but had no effect on that of the *NIS* gene (Fig. 4A).

Figure 2

Effects of MEK and Akt inhibitors in FTC-133 and BCPAP cells. (A) Effects on ERK and Akt phosphorylation. Western blot analysis of phospho-ERK and phospho-Akt expression in FTC-133 and BCPAP cell lines treated with MEK inhibitor U0126 at 10 μ M and the Akt inhibitor IV (B2311) at 0.5 μ M. Total ERK and Akt were used as internal controls. Numbers reported in figure indicate mean values $\pm$ s.d. of p-ERK and p-Akt of three different experiments. Each value was normalized with respect to total ERK and Akt levels. An immunoblot representative of three experiments is shown.

(B) Effects on *TSHR* and *NIS* expression in FTC-133 and BCPAP cell lines after treatment with MEK inhibitor U0126 at 10 μ M and Akt inhibitor IV (B2311) at 0.5 μ M for 15 and 30 h. Total RNA was extracted as described in 'Subjects and methods' section. Results are expressed as the mean $\pm$ s.d. of values obtained from at least three different experiments and for endogenous control β -actin is used. Statistical analysis was performed using the Tukey-Kramer multiple comparisons test. * P < 0.05, ** P < 0.01, and *** P < 0.001.

In order to test whether the effects induced by B2311 and U0126 on histone post-translational modifications are specific for *NIS* or *TSHR* genes, the effects on global histone levels were investigated by western blot. As shown in Fig. 4B, histone recovery was lower in FTC-133 than in BCPAP. Nevertheless, it is clear that the only significant effect that we were able to detect was the inhibitory effect of U0126 in BCPAP on H3K4me3 levels. These data indicate that most effects induced by B2311 on FTC-133

and U0126 on BCPAP at the level of histone post-translational modifications of *NIS* and *TSHR* promoter are gene-specific and not related to global effects.

These data clearly demonstrate that in thyroid cancer cell lines post-translational histone H3 modifications at *TSHR* and *NIS* are regulated by MEK and Akt signaling pathways. The effects of this regulation on the *TSHR* gene are fully consistent with current models of epigenetic modulation of transcription. However, this is not true for

Figure 3

Effects of MEK and Akt inhibitors on other thyroid-specific gene expression. Expression of *FOXE*, *NKX2.1*, *PAX8*, and *Tg* expression in FTC-133 and BCPAP cell lines after treatment with MEK inhibitor U0126 at 10 μ M and Akt inhibitor IV (B2311) at 0.5 μ M for 15 and 30 h. Total RNA was extracted as described in 'Subjects and methods' section. Results are expressed as fold of increase/decrease with respect to untreated cells, considered arbitrarily as 1 (horizontal dashed line). Each column indicates the mean $\pm$ s.d. value obtained from at least three different experiments. As endogenous control β -actin is used. Statistical analysis was performed as described in 'Subjects and methods' section. * $P < 0.05$.

NIS gene. This difference, which is particularly evident in the results of our FTC-133 cell assays, adds further support to the hypothesis that these two thyroid-specific genes are subjected to different forms of epigenetic control in thyroid cancers.

Effects of MEK–Akt inhibitors on thyroid cell proliferation

The MTT assay was used to evaluate the effects of U0126 and B2311 (24 and 48 h of exposure) on FTC-133 and BCPAP cell viability. As shown in Fig. 5, each of the

inhibitors significantly reduced the viability of FTC-133 cells (reductions vs controls of $\sim 20\%$ with U0126 and 30% with B2311, after 48 h of exposure). In BCPAP cells, both inhibitors also reduced viability, but the effects were already highly significant ($P < 0.001$) after 24 h of exposure (reductions of 30% with U0126 and 20% with B2311), and the reductions observed at 48 h were larger than those seen in FTC-133 cells (e.g. 35% with U0126 and 45% with B2311) (Fig. 5).

Discussion

Differential alteration of *NIS* and *TSHR* expression in thyroid cancer has been frequently reported. Using an *in vivo* model of normal human thyroid cells, we previously demonstrated that TSH suppression affects *NIS* mRNA levels but not those of *TSHR* (Bruno *et al.* 2005). Characterization of the elements that regulate *TSHR* (Kakinuma *et al.* 1996) and *NIS* (Chieffari *et al.* 2002) expression has stimulated/facilitated attempts to identify the roles these genes play in human thyroid cancer. Most studies have found that *NIS* expression in thyroid cancers is reduced or absent (except for some cases with defective membrane transport) (Kogai & Brent 2012), whereas *TSHR* expression in these tumors is often preserved (Brabant *et al.* 1991, Ohta *et al.* 1991, Lazar *et al.* 1999, Sheils & Sweeney 1999, Tanaka *et al.* 2000, Durante *et al.* 2007; Sponziello, Durante C, Russo D & Filetti S, 2013, unpublished observations). The latter property of most thyroid cancers, combined with the TSH stimulation of *NIS* expression and function, has been and is currently exploited for the TSH-induced radioiodine conventional treatment of differentiated thyroid malignancies (Schlumberger *et al.* 2007, Wartofsky & Van Nostrand 2012).

In this study, striking differences were noted between the *NIS/TSHR* expression patterns observed in the various thyroid cancer cell lines we investigated, under basal conditions and after suppression of tumorigenic signaling pathways. Responses to MEK and Akt inhibitors were influenced in part by the genotypic alterations found in these cell lines. In BCPAP cells, for example, which are characterized by activating *BRAF* mutation (Schweppe *et al.* 2008), inhibition of ERK phosphorylation with U0126 upregulated the expression of *NIS* and *TSHR*. Phosphorylation of ERK has been implicated as a major mechanism underlying the *BRAF*-dependent loss of differentiation reported in many studies (Knauf & Fagin 2009), and our findings are thus consistent with the proposed use of inhibitors like U0126 to treat thyroid cancer (Puxeddu *et al.* 2011). The *TSHR* and *NIS* expression patterns were quite different in FTC-133 cells, which

Figure 4

Effects on histone H3 post-translational modifications of *TSHR* and *NIS* genes in BCPAP and FTC-133. (A) ChIP assays. BCPAP cells were treated with U0126 and FTC-133 with B2311 for 24 h. Bars indicate levels of acetylation at lysines 9 and 14 (top), trimethylation at lysine 4 (middle), and trimethylation at lysine 27 (bottom). NT, not treated. Each bar represents the mean value of three different determinations. For each bar s.d. is not above 15% of each value. (B) Basal histones levels. Western blots were performed as described in 'Subjects and methods' section. DMSO indicates

cell treated by vehicle alone. Numbers below images of H3K9K14ac, H3K4me3, and H3K27me3 indicate mean values $\pm$ s.d. of triplicate evaluation. In order to evaluate levels of post-translational modifications, each number has been obtained by normalization first with β -actin and then with total H3 histone levels (H3). All differences except those between DMSO and U0126 in BCPAP cells are statistically not significant. For each antibody an immunoblot representative of three experiments is shown.

harbor a defective *PTEN* gene that causes activation of the Akt-dependent pathway (Liu *et al.* 2011). In this study, B2311-mediated inhibition of Akt phosphorylation upregulated the expression of *NIS*, but it markedly downregulated that of the *TSHR* gene, which suggests that expression of the latter gene – at least in this cell line – may be associated with the activation of growth-promoting signals. The findings regarding the *TSHR* are limited to only one cell line investigated, FTC-133, and could not be reproduced in other cell lines, because most of them show loss of *TSHR* expression (Pilli *et al.* 2009; Sponziello M, Durante C, Russo D & Filetti S, 2013, unpublished observations), probably as a result of the cells' adaptation to *in vitro* growth conditions (Van Staveren *et al.* 2007). However, the *NIS/TSHR* expression pattern of FTC-133 resembles that observed in thyroid cancer cells *in vivo*, where *TSHR* expression is often preserved or only mildly decreased. Extension of our investigation to other thyroid-specific genes demonstrated a heterogeneous pattern of gene expression, supporting the concept of gene- and cell line-specific behavior, due to the different origin of the thyroid cancer cell lines.

Our hypothesis that the expression of *NIS* and *TSHR* is differentially regulated is also supported by our epigenetic data, especially the results of experiments conducted with FTC-133 cells. In this cell line, under basal conditions and after treatment with the Akt inhibitor, the correlation between *TSHR* mRNA levels and post-translation modifications of histone H3 was in accordance with current knowledge on the effects on gene expression of specific histone modifications (Füllgrabe *et al.* 2011). FTC-133 cells exhibited increased levels of H3K27me3 (which indicates transcriptional silencing) and reduced levels of H3K9K14ac and H3K4me3 (which are transcription-activating marks). In contrast, this is not true for expression of the *NIS* gene (decrease in H3K9K14ac and H3K4me3 levels and increase in H3K27me3 after treatment with the Akt inhibitor). In addition, even in BCPAP cells, H3 histone post-translational modifications resulted partially coherent with the current models of how these epigenetic marks control transcription only in the case of *TSHR*. However, our epigenetic data regarding both *TSHR* and *NIS* indicate that each cell line possesses its own profile, highlighting the extreme variability of this phenotype.

Figure 5

Effects of MEK and Akt inhibitors on FTC-133 and BCPAP cell viability. BCPAP and FTC-133 were treated for 24 and 48 h with U0126 (10 μ M) or B2311 (0.5 μ M), and MTT assays were performed as described in 'Subjects and methods' section. Bars indicate the percentage of viable cells vs controls (untreated cells) and represent means $\pm$ s.d. of three experiments. *** $P < 0.001$.

Thus, a major finding of our study is that in thyroid cancer cells MEK and Akt signaling pathways control post-translational modifications of histones at specific genes. Regulation of these epigenetic marks by signaling pathways important in cancer is an emerging notion. Studies conducted in neural stem cells, for example, have shown that MEK pathway activation modifies the effects of retinoic acid on H3K4me3 levels of various genes (Ekici *et al.* 2008), and the activation of the Akt pathway in MCF-7 breast cancer cells reduces the levels of H3K27me3 through phosphorylation of EZH2 (Bredfeldt *et al.* 2010).

Thus far, the correlation between tumor aggressiveness and histone modifications has been investigated exclusively at the overall only for global (and not gene-specific) histone modifications (Puppini *et al.* 2011). Our data clearly indicate that signaling through the MEK and Akt pathways in thyroid cancer cells exerts a contemporary control on distinct post-translational histone modifications, so that their ultimate effects are gene-specific. It is also important to recall that suppression of the MAPK and PI3K/Akt pathways and inhibition of histone deacetylase

activity have all been shown to induce NIS expression and radioiodine uptake even in certain non-thyroid human cancer cells (Liu & Xing 2012). Finally, in our cell lines, such modifications appear when the expected effects on the cell growth are still limited to an $\sim 30\%$ inhibition of proliferation rate.

As epigenetic marks play major roles in the control of gene expression, further studies are needed to understand how Akt and MEK, as well as other signaling pathways, regulate gene-specific histone post-translational modifications may provide new hints for a better knowledge of mechanisms underlying thyroid tumorigenesis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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