MAPK and SHH pathways modulate type 3 deiodinase expression in papillary thyroid carcinoma

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Abstract

Type 3 deiodinase (DIO3, D3) is reactivated in human neoplasias. Increased D3 levels in papillary thyroid carcinoma (PTC) have been associated with tumor size and metastatic disease. The objective of this study is to investigate the signaling pathways involved in *DIO3* upregulation in PTC. Experiments were performed in human PTC cell lines (K1 and TPC-1 cells) or tumor samples. *DIO3* mRNA and activity were evaluated by real-time PCR and ion-exchange column chromatography respectively. Western blot analysis was used to determine the levels of D3 protein. *DIO3* gene silencing was performed via siRNA transfection. *DIO3* mRNA levels and activity were readily detected in K1 (BRAF^{V600E}) and, at lower levels, in TPC-1 (RET/PTC1) cells ($P < 0.007$ and $P = 0.02$ respectively). Similarly, *DIO3* mRNA levels were higher in PTC samples harboring the BRAF^{V600E} mutation as compared with those with RET/PTC1 rearrangement or negative for these mutations ($P < 0.001$). Specific inhibition of BRAF oncogene (PLX4032, 3 μ M), MEK (U0126, 10–20 μ M) or p38 (SB203580, 10–20 μ M) signaling was associated with decreases in *DIO3* expression in K1 and TPC-1 cells. Additionally, the blockage of the sonic hedgehog (SHH) pathway by cyclopamine (10 μ M) resulted in markedly decreases in *DIO3* mRNA levels. Interestingly, siRNA-mediated *DIO3* silencing induced decreases on cyclin D1 expression and partial G1 phase cell cycle arrest, thereby downregulating cell proliferation. In conclusion, sustained activation of the MAPK and SHH pathways modulate the levels of *DIO3* expression in PTC. Importantly, *DIO3* silencing was associated with decreases in cell proliferation, thus suggesting a D3 role in tumor growth and aggressiveness.

Key Words

- ▶ type 3 deiodinase, papillary thyroid carcinoma
- ▶ MAPK genetic alterations
- ▶ Sonic Hedgehog pathway

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Introduction

Thyroid hormone (TH) influences a wide variety of biological processes including the balance between cell proliferation and differentiation. TH homeostasis is

critically regulated by the synchronized activity of the iodothyronine deiodinases. Type 1 (*DIO1*, D1) and type 2 deiodinases (*DIO2*, D2) catalyze the conversion of the

pro132-hormone thyroxine (T_4) into the biologically active form triiodothyronine (T_3) via outer ring deiodination. In contrast, type 3 iodothyronine deiodinase (*DIO3*, D3) catalyzes the inactivation of T_4 and T_3 via inner ring deiodination (Maia et al. 2011). Several studies have shown an association between thyroidal status and tumor pathogenesis (Pinto et al. 2011, Kim & Cheng 2013) while several reports have documented changes in the expression of deiodinases in benign and malignant tumors (Huang et al. 2001, Murakami et al. 2001, de Souza Meyer et al. 2005, Dentice et al. 2007, 2012, Meyer et al. 2007).

D3, a known fetal protein, is reactivated in human neoplasias and has been associated with tumor behavior. The upregulation of *DIO3* gene occurs at the transcriptional level, and it might be driven by disruption of several signaling pathways such as MAPK, transforming growth factor beta (TGFB), sonic hedgehog (SHH) and B-catenin (Dentice & Salvatore 2011). Increased levels of D3 were demonstrated in mouse and human malignant basal cell carcinoma (BCC). In BCC, the *DIO3* induction occurs via activation of the SHH/GLI pathway and is associated with increased levels of cyclin D1 and keratinocyte proliferation (Dentice et al. 2007). High levels of *DIO3* mRNA also occur in human intestinal adenomas and carcinomas (Dentice et al. 2012). Interestingly, the activating TH enzyme, D2, was downregulated by SHH and B-catenin activation. Thus, this opposite but convergent action of D2 and D3 enzymes seems to contribute to the local TH attenuation, leading to proliferative effects.

Papillary thyroid carcinoma (PTC) is the most common malignant thyroid tumor, occurring in 85–90% of cases (Pellegri et al. 2013). Aberrant activation of MAPK pathway is a hallmark in PTC. The *BRAF*^{V600E} mutation is the most common genetic event, observed in ~50% of PTC, while RET/PTC rearrangement occurs in ~20% and RAS mutations in 10–15% of cases (Kimura et al. 2003, Fagin & Mitsiades 2008). Increased levels of D3 were shown in PTC and associated with advanced disease (Romitti et al. 2012). Augmented D3 levels were also observed in follicular thyroid carcinoma but not in medullary or anaplastic thyroid carcinoma samples. The reasons for these distinct patterns of D3 expression is still lacking.

In the present study, we sought to determine the signaling pathways involved in *DIO3* upregulation in PTC as well as to investigate whether *DIO3* induction is associated with neoplastic thyroid cell proliferation.

Material and methods

Cell culture

Studies evaluating *DIO3* gene regulation were performed in two human PTC-derived cell lines, which endogenously express the *DIO3* gene: K1 cell line (European Collection of Cell Cultures; ECACC, Porton Down, Salisbury, UK), which carries the *BRAF*^{V600E} mutation, and TPC-1 cells (kindly provided by Dr J A Fagin to Dr E T Kimura, Memorial Sloan Kettering Cancer Center, New York, NY; Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, NY, USA) harboring the RET/PTC1 rearrangement (Geraldo et al. 2012). The origin in human PTC and gene expression profile have already been demonstrated for both cell lines (Schweppe et al. 2008, Saiselet et al. 2012). K1 cells were grown in DMEM: Ham's F12:MCDB 105 (2:1:1 (Life Technologies, Invitrogen)) plus 2 mM glutamine and 10% fetal bovine serum (FBS) (Life Technologies, Invitrogen). TPC-1 cells were grown in DMEM containing 10% FBS. Additionally, we used a human medullary thyroid carcinoma (MTC) cell line carrying the *RET* C634Y mutation – TT cells (Carlomagno et al. 1995; American Type Culture Collection, ATCC, Manassas, VA, USA) – to determine the effect of SHH on *DIO3* reactivation. TT cells were grown in RPMI (Life Technologies, Invitrogen) medium supplemented with 10% FBS. All experiments were performed between passages 15–25 for K1 cells, 20–25 for TPC-1 cells and 10–15 for TT cells. All cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air, and the culture medium was changed three times a week. Experiments were performed in cell cultures at 60–80% confluence. All analyses were performed in triplicate in at least three independent experiments.

Human PTC samples

Neoplastic tissues were collected from 25 unselected PTC patients who underwent thyroidectomy at our institution. Non-tumor thyroid tissues adjacent to tumor were also collected. All PTC and normal tissue samples ($n=9$) were examined by a pathologist to exclude the presence of necrosis and/or inflammation. Tumors were histologically classified according to WHO recommendations (Hedinger et al. 1989). The Ethical Committee of the Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil, approved the study.

BRAF^{V600E} mutation and RET/PTC rearrangement analysis

DNA was extracted from 10-μm slides of paraffin-embedded tissue blocks, using the Magnesil Genomic

Fixed Tissue System (Promega Corporation), according to the manufacturer's instructions. The *BRAF* exon 15 was amplified by PCR using specific oligonucleotides (Supplementary Table S1, see section on supplementary data given at the end of this article). For PCR amplification, we used 50–100 ng/μl of DNA in a reaction mix (25 μl) containing 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM MgCl₂, 0.2 mM dNTPs, 0.2 mM of each primer and 1.25 U Platinum Taq DNA polymerase (Invitrogen). *BRAF*^{V600E} mutation genotyping was performed by direct sequencing (Life Technologies, Applied Biosystems).

For RET/PTC1 detection, total RNA was extracted from PTC samples using the Trizol Reagent, and cDNA was generated using the Super Script III First-Strand Synthesis System (Life Technologies, Invitrogen, Carlsbad, CA, USA). Detection of RET/PTC1 rearrangement was performed by RT-PCR. We used the forward primer for *H4* gene and the reverse primer for the *tyrosine kinase* domain

of *RET* (Supplementary Table S1). PCR conditions were performed according to a previously described protocol (Sapio et al. 2007). Afterwards, 10 μl of the PCR product were analyzed by electrophoresis in a 1.5% agarose gel. TPC-1 cells were used as a positive control. Positive samples were subjected to direct sequencing to confirm the presence of RET/PTC rearrangement.

Immunohistochemistry studies

D3 expression was evaluated by immunohistochemistry studies in matched normal thyroid tissue, primary PTC tumor and lymph node metastases from two PTC patients ($n=18$ and $n=19$; Table 1). Immunohistochemistry analysis of D3 was performed on 6-μm sections of formalin-fixed and paraffin-embedded tissues. The immunohistochemical technique comprised tissue deparaffinization and rehydration, antigenic recovery, inactivation

Table 1 Clinical and oncological features of the patients with PTC

Patient number	Sex	Age at diagnosis (years)	Tumor size (cm)	Histology	Stage (TNM)	Sites of metastasis	<i>BRAF</i> ^{V600E}	RET/PTC1
1	M	50	1.5	Classic type	III	Cervical lymph nodes	–	–
2	M	22	1.3	Classic type	II	Cervical lymph nodes; lung	–	+
3	M	22	2.3	Follicular variant	I	Cervical lymph nodes; lung	–	–
4	F	33	1.0	Classic type	I	Cervical lymph nodes	–	+
5	F	27	6.4	Classic type	II	Cervical lymph nodes; lung	+	–
6	F	36	2.5	Follicular variant	I		–	–
7	F	53	0.8	Classic type	I	Cervical lymph nodes	+	–
8	F	42	2.7	Classic type	I		+	–
9	F	50	2.5	Classic type	II	Cervical lymph nodes	+	–
10	F	47	2.3	Classic type	II		–	–
11	M	50	3.8	Classic type	III	Cervical lymph nodes	+	–
12	F	61	8	Classic type	IV	Cervical lymph nodes; lung	–	–
13	F	42	8	Classic type	I	Cervical lymph nodes; conjunctive tissue and skeletal muscle	+	–
14	F	10	10.5	Classic type	I	Cervical lymph nodes	+	–
15	F	18	3.5	Classic type	I	Skeletal muscle of the neck	–	–
16	F	55	3	Follicular variant	IV	Cervical lymph nodes	+	–
17	F	11	4.7	Classic type	II	Cervical lymph nodes	–	–
18	M	27	3	Follicular variant	I	Cervical lymph nodes	–	–
19	F	53	2.5	Classic type	III	Cervical lymph nodes	+	–
20	F	51	2.5	Follicular variant	II	Cervical lymph nodes	+	–
21	M	35	1	Classic type	I	Cervical lymph nodes	+	–
22	F	49	0.9	Follicular variant	I		–	–
23	F	30	2.5	Classic type	I		+	–
24	F	21	0.8	Classic type	I	Cervical lymph nodes	–	–
25	F	31	1	Follicular variant	I		+	–

of endogenous peroxidase and blockage of unspecific reactions. D3 anti-rabbit polyclonal antibody (Novus Biologicals, Littleton, CO, USA) was incubated overnight at a temperature of 4°C, at dilution of 1:50 (the same antibody concentration was also used for positive control), followed by subsequent incubation with biotinylated secondary antibody, streptavidin–HRP conjugate (LSAB; Dako, Carpinteria, CA, USA) and diaminobenzidine tetrahydrochloride (Kit DAB; Dako). Placenta tissue was used as a positive control. The negative control was obtained by omission of the primary antibody. The slides were examined using an Olympus BX51 microscope with an Olympus QColor 5 camera. The QCapturePro software was used to capture the images.

Real-time PCR

Total RNA was extracted from K1 and TPC-1 cells using the RNeasy minikit (Qiagen). Trizol Reagent was used for total RNA extraction from PTC samples and surrounding thyroid tissues. One microgram of RNA was reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR (Life Technologies, Invitrogen), following the manufacturer's protocol for the oligo (dT) method. RT-qPCR experiments were performed in a 7500 Fast Real-Time PCR System Thermal Cycler with 7500 FAST System Sequence Detection 1.4 Software (Life Technologies, Applied Biosystems). Experiments were performed by real-time monitoring of the increase in fluorescence of SYBR Green dye. The oligonucleotides used are described in [Supplementary Table S1](#), see section on [supplementary data](#) given at the end of this article. Each sample was assayed in triplicate, and a negative control was included in each experiment. Standard curves representing five-point serial dilution of cDNA were analyzed and used as calibrators of the relative quantification of product generated in the exponential phase of the amplification curve. The R^2 was greater than 0.99, while the amplification efficiency was higher than 98%. Quantification of *DIO3*, *DIO2* and *GAPDH* cDNA were performed by relative quantification using the comparative $\Delta\Delta CT$ method and expressed relative to the reference gene (cyclophilin A). Changes in gene expression were expressed as relative fold difference (n -fold change) or as arbitrary units (AUs).

Inhibition of MAPK and SHH signaling

To evaluate the effect of MAPK signaling activation on *DIO3* induction in K1 and TPC-1 cell lines, we performed studies using specific inhibitors to the signaling effectors

MEK (U0126: 10–20 μ M; Sigma–Aldrich), p38 (SB203580: 10–20 μ M; Sigma–Aldrich) and *BRAF* mutated (PLX4032: 3 μ M; Selleck Chemicals, Houston, TX, USA). Additionally, to assess the role of the SHH pathway on *DIO3* regulation, we used the specific inhibitor of the smoothed (a SHH signaling effector), cyclopamine (10 μ M, Sigma–Aldrich). The recombinant human SHH (1 μ g/ml, R&D Systems, Minneapolis, MN, USA) was used to induce SHH activation in TT cells. Controls were incubated with medium + vehicle (1% DMSO, Sigma–Aldrich). Cells were incubated during 24 h and then were harvested and processed for total RNA or total protein extraction.

D3 activity assay

D3 activity was determined in PTC cells by ion-exchange column chromatography ([Wajner et al. 2011](#)). After concluding the experiments, 300 μ l of medium was collected, and the reaction was stopped with 200 μ l horse serum and 100 μ l 50% TCA, which was followed by centrifugation at 12 000 g for 2 min to precipitate the non-metabolized (125I) T_3 . The supernatant was used to determine (125I) T_2 and (125I) T_1 levels. The Sephadex LH-20 column was equilibrated with 0.1 M HCl, and an equal volume of 0.1 M HCl was added to 500 μ l samples and then mixed. Stepwise elution was performed by successive application of 2 $\times$ 1 ml of 0.1 M HCl (for 125I release), 6 $\times$ 1 ml of 0.1 M NaOH–ethanol (8:1 v/v (125I) for T_1 release) and 4 $\times$ 1 ml of 50% ethanol in 0.1 M NaOH (1:1 v/v (125I) for T_2 release). The 1-ml fractions were collected and counted for radioactivity. The D3 activity was calculated by multiplying the fractional conversion by the T_3 concentration in the media and expressed as T_3 inactivation (fmol/mg protein per 24 h).

Western blot analysis

Cultured cells were lysed and prepared for western blot analysis, as previously described ([Fuziwara & Kimura 2014](#)). Briefly, 30–50 μ g protein of each sample was fractionated by 8–12% SDS–PAGE and blotted onto an Immobilon PVDF membrane (Millipore, Billerica, MA, USA). Non-specific binding sites were blocked by incubation with 5% nonfat dry milk in Tris-buffered saline 0.1% Tween-20. The following primary antibodies were used: anti-D3 (1:400; Novus Biologicals), anti-ERK1/2 (1:400; Santa Cruz Biotechnology), anti-phospho-ERK1/2 (1:400; Santa Cruz Biotechnology), anti-p38 (1:500; Cell Signaling), anti-phospho-p38 (1:200; Santa Cruz Biotechnology), anti-cyclin D1 (1:400; Santa Cruz

Biotechnology), anti-Gli1 (1:400; Cell Signaling Technology), anti- α -tubulin B7 (1:500; Santa Cruz Biotechnology) and anti- β -actin (1:10 000; Sigma-Aldrich). The antigen-antibody complexes were visualized using HRP-conjugated secondary antibody and an enhanced chemiluminescence system (GE Healthcare, Pittsburgh, PA, USA). Expression was quantified using image densitometry with ImageJ analysis software.

siRNA transfection

siRNA studies were performed to evaluate the specific effects of *DIO3* inhibition on cell proliferation. The siRNAs were as follows: Silencer Select *GAPDH* siRNA (#4390849, Life Technologies, Ambion Inc., Austin, TX, USA) was used as positive control; Silencer Select Negative Control (#4390843, Life Technologies, Ambion Inc.) and Silencer Pre-designed *DIO3* siRNA (#AM16708, Life Technologies, Ambion Inc.) were used in the experiments. Transfection studies were performed using Lipofectamine RNAiMAX reagent, according to the manufacturers' instructions (Life Technologies, Invitrogen). A total of 15×10^4 cells/well (K1 and TPC-1) were plated in six-well plates and transfected with 40 pmol of *GAPDH* siRNA, 100 pmol of silencer negative and 100 pmol of *DIO3* siRNA. All analyses were performed in triplicate and in at least three independent experiments.

Cell proliferation assays

Absolute cell number count and flow cytometry were performed to evaluate cell proliferation. Initially, 15×10^4 cells/well (K1 and TPC-1) were plated in six-well plates, transfected with 100 pmol of *DIO3* siRNA and incubated for 48 h. The cells were trypsinized, and the absolute number was counted using the Neubauer chamber. To evaluate the effect of *DIO3* expression on the cell cycle status, K1 cells were incubated with *DIO3* siRNA. After 48 h, cells were washed with PBS and then resuspended in 50 μ g/ml propidium iodide and 0.1% Triton X-100 in sodium citrate solution. Cells were incubated on ice for at least 15 min. Marked cells were analyzed using Attune NxT Acoustic Focusing Cytometer (Life Technologies, Applied Biosystems). The data generated were analyzed using the FlowJo software. All experiments were performed in triplicate.

Statistical analysis

DIO3 mRNA was expressed as AUs or fold, while D3 activity as mean $\pm$ s.d. The number of cells in each cell

cycle stage is shown as frequency. To compare D3 levels among the groups, we used *t*-test or one-way ANOVA. χ^2 test was used to compare the differences in the proportion of cells in different stages of cell cycle. The SPSS 18.0 and Prism 5.0 software were used for all analyses, and $P < 0.05$ was considered statistically significant.

Results

MAPK activation induces *DIO3* levels in PTC cell lines

To evaluate the role of MAPK activation in *DIO3* regulation, experimental studies were performed in two distinct human PTC cell lines, K1 cells carrying the *BRAF*^{V600E} mutation and TPC-1 cells harboring the RET/PTC1 rearrangement. We observed that, although *DIO3* expression was readily detected in both cell lines (Fig. 1A), the levels of *DIO3* mRNA and activity were significantly higher in K1 cells as compared to those observed in TPC-1 cells (0.32 vs 0.11 AU, $P = 0.007$; 14.9 vs 8.1 fmol/mg.prot.24 h, $P = 0.02$ respectively; Fig. 1A and B).

Next, we evaluated the oncogenic effects of *BRAF*^{V600E} mutation on *DIO3* reactivation. The incubation of K1 cells with the specific *BRAF*-mutated inhibitor, PLX4032 (3 μ M), decreased ERK phosphorylation in $\sim 30\%$ (Fig. 2A) as well as the *DIO3* mRNA levels (~ 2.2 -fold, $P < 0.001$; Fig. 2B). Similar results were obtained with higher PLX4032 doses, indicating that PLX4032-mediated decreases in ERK phosphorylation is not dose dependent (not shown). On the other hand, the incubation of K1 cells with increasing doses of MEK inhibitor (U0126, 10–20 μ M) for 24 h resulted in a dose-dependent reduction in ERK phosphorylation (Fig. 3A) and in *DIO3* expression (10- to 12.5-fold, $P < 0.001$; Fig. 3B). Likewise, the incubation of K1 cells with SB203580, a specific inhibitor of p38 protein, caused a dose-dependent reduction of phospho-p38 levels

Figure 1

DIO3 mRNA levels (A) and activity (B) were readily detected in both PTC cell lines, K1 and TPC-1, and were significantly higher in K1 cells compared to TPC-1 cells. * $P < 0.007$; ** $P = 0.02$.

Figure 2

Incubation of K1 cells with the *BRAF*-mutated specific inhibitor, PLX4032 (3 μ M), for 24 h led to a reduction in the ERK phosphorylation (A) and *DIO3* mRNA (B). * $P < 0.001$.

(Fig. 3C) as well as with decreases of *DIO3* expression (~ 2 -fold, $P < 0.001$; Fig. 3D).

Similar experiments were performed in TPC-1 cells. We observed a dose-dependent reduction in MEK and p38 phosphorylation in TPC-1 cells treated with specific inhibition of MEK (U0126) or p38 (SB203580) (Fig. 3E and G respectively). *DIO3* expression was reduced in a dose-dependent fashion in the presence of both inhibitors (2.2- to 7-fold for U0126 and 2- to 3-fold for p38 respectively, $P < 0.001$; Fig. 3F and H).

We also investigated the effect of MAPK genetic alterations on the *DIO3* levels in 25 human PTC samples. Clinical and oncological features are detailed in Table 1.

Mean age of patients was 37.04 ± 14.7 years, and 76% were women. The median tumor size was 2.5 cm (0.8–10.5); 13 patients (52%) had lymph node metastasis, while 6 (24%) had distant metastasis at diagnosis. In total, 13 (52%) out of the 25 were positive for the *BRAF*^{V600E} mutation, 2 (8%) carried RET/PTC1 rearrangement and 10 (40%) were negative both genetic alterations. As previously shown (Romitti et al. 2012), *DIO3* mRNA levels were significantly increased in PTC samples as compared with the levels observed in normal thyroid tissue ($P < 0.001$). Samples harboring *BRAF*^{V600E} mutation displayed higher levels of *DIO3* expression as compared with those observed in samples with RET/PTC1 rearrangement or negative for *BRAF*^{V600E} and RET/PTC1 alterations (7.97- vs 5.86- vs 5.85-fold respectively, $P < 0.001$; Fig. 4). It is interesting, however, that the highest level of *DIO3* expression was found in a sample negative for *BRAF* and RET/PTC1 (patient 18, Table 1).

To gain insights on D3 expression in PTC, we undertook immunohistochemical studies to evaluate D3 levels in PTC metastasis samples. Matched paraffin-embedded thyroid tumor and metastatic lymph node tissues were obtained from two PTC patients ($n = 18$ and $n = 19$; Table 1). Remarkably, in both cases, the intensity of D3 staining was similar in tumor and metastatic tissue, indicating that D3 upregulation is a characteristic feature of this malignant neoplasia, occurring also in metastatic sites (Fig. 5A, B, C, D).

Figure 3

MEK1 inhibition by U0126 incubation in K1 cells significantly blocked the phosphorylation of the ERK pathway (A) as well as caused a dose-dependent reduction in the *DIO3* levels (B). The inhibition of p38 protein

by SB203580 inhibitor in K1 cells inhibited p38 phosphorylation (C) while reduced the *DIO3* transcripts (D). Similarly, the reduction of pERK (E) and p38 (G) reduced the *DIO3* levels in TPC-1 cells (* $P < 0.01$).

Figure 4

DIO3 mRNA was significantly increased in PTC samples as compared with thyroid normal tissue. Samples harboring *BRAF*^{V600E} mutation ($n=13$) had higher levels of *DIO3* expression compared with samples presenting RET/PTC1 rearrangement ($n=2$) or those negative for *BRAF* or RET/PTC1 mutations ($n=10$) ($P<0.001$). The levels of *DIO3* mRNA of PTC samples are expressed as the fold change relative to the reference levels of normal thyroid samples ($n=9$), represented as 1.

Blockage of SHH pathway prevents *DIO3* upregulation in PTC

Next, we evaluated whether the SHH pathway and its downstream effector, GLI1, could be involved on *DIO3* regulation in PTC. The SHH signaling was blocked using cyclopamine (10 μ M). We observed a reduction in the GLI1 protein (Fig. 6A and C for K1 and TPC cells respectively), followed by a marked decrease in the *DIO3* levels in K1 (~12-fold, $P<0.001$; Fig. 6B) as well as in TPC-1 cells (~2.5-fold, $P<0.001$; Fig. 6D).

To identify whether MAPK and SHH cooperate in promoting *DIO3* induction in PTC, we evaluated GLI1 protein expression under MEK and p38 proteins inhibition. We observed a reduction in GLI1 levels in K1 and TPC-1 cells after MAPK blockage with U0126 (Fig. 6E and G) or SB203580 (Fig. 6F and H), indicating that these signaling pathways might cooperate on *DIO3* regulation in PTC. Of note, however, the treatment of cells with cyclopamine has no effect on ERK or P38 phosphorylation (data not shown).

We then sought to explore the role of SHH signaling pathway on *DIO3* reactivation. We have previously shown that MTC, a C-cell originated tumor, does not express D3 enzyme (Romitti et al. 2012). Low levels of *DIO3* mRNA were detected in MTC-derived TT cells while a marked increase in *DIO3* expression was observed in recombinant SHH-treated TT cells (2.5-fold, $P<0.001$; Fig. 6I). In

contrast, recombinant SHH treatment significantly decreased *DIO2* mRNA levels in TT cells (2.6-fold, $P<0.001$; Fig. 6J). It is intriguing, however, that recombinant SHH has no further effect on the already upregulated *DIO3* expression in K1 and TPC-1 cells (data not shown).

DIO3 silencing is associated with reduction in PTC cell proliferation

To evaluate whether the *DIO3* upregulation induces proliferation of neoplastic thyroid cells, we silenced the *DIO3* gene in both PTC cell lines (K1 and TPC-1). The experiments were performed using a *DIO3*-specific siRNA (100 pmol) and *GAPDH* siRNA (40 pmol) as a positive control. K1 and TPC-1 cells were transfected with the siRNAs and maintained for 48 h. The efficiency of siRNA transfection was established by the efficiency in silencing *GAPDH* gene (inhibition of 95% in K1 and ~90% in TPC-1 cells, $P<0.001$). *DIO3* gene knockdown resulted in a ~90% blockage of *DIO3* transcripts in both cell lines ($P<0.001$; Fig. 7A and C). Interestingly, *DIO3* inhibition was associated with significant reduction in the absolute cell number, as compared with control (~30%, $P<0.01$; Fig. 7B and D) and decreased levels of cyclin D1 (Fig. 7E). Accordingly, further experiments showed that *DIO3* silencing was associated with augmented proportion of cells in the G1 phase and a proportional reduction in the percentage of cells in S and G2 phases of cell cycle (~30%, $P<0.005$; Fig. 7F).

Discussion

In the present study, we have demonstrated that genetic alterations in the MAPK pathway effectors, such as *BRAF*^{V600E} mutation and RET/PTC1 rearrangement, increase *DIO3* levels. The SHH pathway is also involved in *DIO3* upregulation once its signaling inhibition significantly reduces the *DIO3* expression. Interestingly, siRNA-mediated *DIO3* gene silencing decreases cyclin D1 levels while increasing the proportion of cells in the G1 phase of the cell cycle, reducing the proliferation of malignant thyroid cells.

A potential role of D3 in tumorigenesis has been postulated due to *DIO3* upregulation in several benign and malignant tumors (Pallud et al. 1999, Huang et al. 2005, Dentice et al. 2007, 2012, Romitti et al. 2012). Studies performed in primary cultures of human fibroblasts showed that the *DIO3* gene is induced by TGFB via SMAD and MAPK-dependent pathway (Huang et al. 2005). PTCs are known for carrying genetic alterations

Figure 5

Representative micrographs of D3 brown-dark immunohistochemical staining samples of human PTC (A, B and C) and lymph node metastasis (B, C and D) tissues from two PTC patients ($n = 18$, A and B, and $n = 19$,

C and D). Matched paraffin-embedded thyroid tumor and metastatic lymph node tissues showed similar intensity of D3 staining. Black bars represent 400 $\times$ magnification.

that lead to an aberrant and constitutive activation of MAPK pathway (Romitti *et al.* 2013). $BRAF^{V600E}$ is an oncogenic protein with markedly elevated kinase activity that overactivates the MAPK pathway, especially ERK signaling transduction (Xing 2005, Chakravarty *et al.* 2011). Here, we show that $BRAF^{V600E}$ mutation is associated with the highest levels of *DIO3* expression, as compared with RET/PTC1 (Fig. 1A and B), which seems to be mediated by the stronger ERK phosphorylation induced by this oncogene (Fig. 3A and B). On the other hand, we also observed that the p38 inhibition led to a more pronounced decrease in the *DIO3* levels in TPC-1 than in K1 cells (Fig. 3G and H; 3E and F respectively), thus suggesting that RET/PTC1 rearrangement induces the *DIO3* levels mainly through this pathway. These observations are in agreement with previous studies which showed that RET/PTC1 rearrangement exerts its effects mainly by p38 phosphorylation (Hayashi *et al.* 2000, Mariggio *et al.* 2007). Consistently, we also observed a stronger induction of *DIO3* expression in human PTC samples carrying the $BRAF^{V600E}$ mutation, as compared to those harboring the RET/PTC1 or none of these genetic alterations (Fig. 4). Interestingly,

similar D3 upregulation was observed in patient-matched tumor and lymph node metastasis, suggesting that the molecular mechanism responsible for D3 upregulation also occurs in the metastatic sites.

SHH signaling is critical for embryogenesis and other cellular processes such as proliferation and differentiation (Lum & Beachy 2004). Disruption in SHH signaling results in various human diseases and seems to contribute to neoplastic processes. The SHH reactivation occurs in up to 25% of human tumors and has been associated with *DIO3* induction (Dentice *et al.* 2007, Aw *et al.* 2014). Here, we show that the SHH signaling also modulates *DIO3* expression in PTC cells. The use of the SHH pathway inhibitor, cyclopamine, promoted a marked decrease in *DIO3* transcripts, indicating a direct effect of SHH/GLI1 signaling on gene regulation (Fig. 6A, B, C, D). Moreover, recombinant SHH treatment induced the *DIO3* expression in TT cells (Fig. 6I). Interestingly, the blockage of the MAPK pathway, using MEK or p38 inhibitors, reduced the levels of GLI1, thus suggesting that the *DIO3* expression in PTC might be regulated by cooperation between the MAPK and SHH pathways, as previously demonstrated in other

Figure 6

Incubation of K1 and TPC-1 cells with a chemical inhibitor of SHH pathway, cyclopamine (10 μ M), diminished GLI1 protein (A and C) while decreased the *DIO3* levels in both cells (B and D respectively; * P <0.001). Inhibition of MAPK proteins, MEK and p38, reduced the levels of GLI1 protein in K1 and

TPC-1 cells (E, F, G and H). Incubation with 1 μ g/ml of recombinant SHH induced *DIO3* mRNA (I) in TT cells while reduced the *DIO3* levels (J; * P <0.001).

pathological conditions (Ji *et al.* 2007, Madhala-Levy *et al.* 2012). The oncogenic effects of the gene *KRAS* in pancreatic cancer cells are mediated by SHH/GLI1 activation, and the suppression of GLI activity led to selective attenuation in the oncogenic activity of mutant *KRAS*-expressing cells (Ji *et al.* 2007). Of interest, two recent

studies have evaluated the efficacy of the SHH inhibitor in patients with locally advanced or metastatic BCC (Sekulic *et al.* 2012, Tang *et al.* 2012). The treatment was associated with significant response rate in patients with metastatic tumors as well as in patients with locally advanced BCC (30 and 43% respectively).

Figure 7

DIO3 knockdown resulted in 90% blockage of *DIO3* transcripts in K1 and TPC-1 cells (A and C respectively; * P <0.001). The reduction in *DIO3* levels was also associated with a significant reduction in the absolute cell number

compared with controls (B and D; ** P <0.01). The *DIO3* silencing in K1 cells was associated with reduction in cyclin D1 protein (E) while increased the proportion of cells in G1 phase of cell cycle (F; *** P <0.005).

Previous studies have indicated that changes in the TH status might interfere with tumor pathogenesis. Clinical hypothyroidism seems to be a risk factor for several neoplasias such as liver cancer, thyroid malignancies, high-grade glioblastomas and human breast cancer (Reddi *et al.* 2007, Polyzos *et al.* 2008, Hassan *et al.* 2009, Angelousi *et al.* 2012). In contrast, increased TH levels have been associated with higher risk of esophageal, thyroid, breast and ovarian cancer development (Turkylmaz *et al.* 2005, Rasool *et al.* 2014, Kunjumohamed *et al.* 2015). Changes in the intracellular TH concentration due to alterations in the deiodinases status seem to be critical for modulating cell proliferation and differentiation. Studies in clear cell renal cell carcinoma (ccRCC) have shown that the loss of *DIO1* expression, mediated by miR-224 induction, resulted in diminishing intratumoral T₃ concentration which contributes to the intracellular hypothyroidism observed in ccRCC (Boguslawska *et al.* 2011). Additionally, experimental studies in BCC and in colon tumor cells have shown that the increase in the *DIO3* levels accompanied of *DIO2* reduction would be associated with the induction of cell proliferation, by overexpression of cyclin D1. Accordingly, *DIO3* knock-down caused a five-fold reduction in the growth of xenograft tumor, while the T₃ addition promoted differentiation (Dentice *et al.* 2007, 2012). Nevertheless, the mechanisms underlying D3 and/or hypothyroidism induction of proliferation are still open to debate. Previous studies have shown that T₃ is involved in regulation of cyclin D1 expression (Nygård *et al.* 2003, Garcia-Silva & Aranda 2004, Furumoto *et al.* 2005, Perri *et al.* 2014). In embryonic carcinoma cells, T₃ treatment decreases the proliferation rate by arresting cells in the G1 and S phases while downregulates E2F1, both key regulators of proliferation. These T₃-mediated effects depend on active TRs (Nygård *et al.* 2003). Accordingly, studies in mouse harboring a dominantly negative mutant TH receptor (TRβPV/PV) suggest that the absence of TRβ, and consequent lacking T₃ signaling, induces cyclin D1 expression, leading to the activation of the CDK/retinoblastoma (RB)/E2F pathway that mediates, at least in part, the aberrant proliferation of thyroid cells (Furumoto *et al.* 2005). In this study, we show that the siRNA-mediated *DIO3* gene attenuation decreases the number and cyclin D1 levels (Fig. 7A, B, C, D, E), followed by a partial G1 phase cell cycle arrest (Fig. 7F). These results further support a pivotal role of *DIO3* overexpression in neoplastic cell proliferation.

In conclusion, we have demonstrated that *DIO3* expression is modulated by MAPK genetic alterations in

PTC. SHH pathway activation might also be involved in *DIO3* upregulation by a signaling cooperation with MAPK pathway. Finally, the reduction on cell proliferation rate in D3-depleted PTC cells supports the hypothesis that the decrease in intracellular TH levels might be associated with tumor growth and aggressiveness.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/ERC-15-0162>.

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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