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ORIGINAL PAPER

In thyroid cancer cell lines expression of periostin gene is controlled by p73 and is not related to epigenetic marks of active transcription

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Abstract
Background Periostin expression is a feature of the epithelial-mesenchymal transition, which occurs during cancer progression. Previous reports indicate that periostin expression is related to tumour aggressiveness.
Methods In order to identify mechanisms regulating periostin expression in thyroid cancer, a panel of continuous thyroid cancer cell lines was investigated. Levels of posttranslational modifications of the H3 histone were investigated by chromatin immunoprecipitation. Moreover, treatment of cell lines with deacetylase inhibitors and transfection experiments were performed.
Results Our insights show that levels of H3 histone acetylated at lysines 9 and 14 (which are epigenetic marks of active transcription) are not related to periostin mRNA levels. Moreover, treatment of WRO and FRO thyroid cancer cell lines with the deacetylase inhibitor tricostatin A (TSA) or suberoylanilide hydroxamic acid (SAHA) increases levels of acetylated H3 histone to periostin promoter however, unpredictably, reduces periostin mRNA levels. Interestingly, treatment of WRO cells with either TSA or SAHA increases levels of the H3 histone trimethylated at lysine 4, which is a different epigenetic mark of active transcription. Instead, data obtained by cell transfection indicate that ΔNp73, a member of p53 family selectively expressed in thyroid carcinomas, plays a role in activating periostin gene expression.
Conclusions Levels of epigenetic marks of active transcription do not contribute to regulation of periostin gene expression. The ΔNp73 effects suggest a novel molecular mechanism involved in thyroid cancer progression.

Keywords Thyroid tumours · Periostin · Histone acetylation · Gene expression · p73

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1 Introduction

Periostin, also known as osteoblast-specific factor-2 (Osf2), is a 90 kDa secreted protein, first identified in bone where regulates osteoblast adhesion and spreading. [1, 2]. This protein is a ligand for vß3 and vß5 integrins, thereby regulating integrin-dependent cell adhesion and motility [3]. Periostin plays a role in formation and structural maintenance of bones and teeth [4] and is involved in heart development [5].

Periostin contains a fourfold repeat structure of about 150 amino acids with a certain degree of homology with the mammalian protein βig-h3 [6], which promotes the adhesion and spreading of fibroblasts in response to transforming growth factor-β (TGF-β [7]. Periostin also interacts with several extracellular matrix proteins such as fibronectin, tenascin-C, collagen V, and periostin itself [6].

Periostin has been recently revealed to play an important role in tumour growth, angiogenesis, invasiveness, and metastasis [8 and references herein]. Certainly, in human breast cancer periostin favours tumour progression and angiogenesis through the up-regulation of vascular endothelial growth factor 2 [9]. In oral squamous carcinoma periostin is frequently overexpressed and may promote invasion and angiogenesis [10]. In pancreatic tumours, periostin overexpression correlates with an aggressive tumour behaviour [11]. High periostin levels in serum of non-small cell lung carcinoma patients correlate with poor clinical outcome and survival [12]. Noteworthy, periostin contributes to the epithelial-mesenchymal transition (EMT), a phenomenon occurring in various carcinomas, which enables epithelial cancer cells to acquire invasive and metastatic potential [13]. Undeniably, ectopic expression of periostin into 293 T cells promotes invasiveness activity through induction of EMT [14].

Thyroid cancer is one of the most frequent endocrine malignancies. Well differentiated thyroid carcinomas are efficiently treated by surgery and radioiodine, while poorly differentiated thyroid carcinomas (10–20% of total) are very resistant to chemotherapy [15]. In differentiated thyroid cancer most frequent genetic alterations results in overactivation of the RAS/RAF/ERK pathway [16]. Other molecular abnormalities occurring in thyroid cancer lead to the disruption of functions related to the p53 tumour suppressor gene [17]. These alterations include p53 mutation in poorly differentiated thyroid carcinomas and overexpression of p53 family members with dominant negative functions (TAp63 and ΔNp73) in well differentiated thyroid carcinomas [18].

We have recently shown that periostin is expressed in papillary thyroid carcinomas (PTC) and that higher periostin expression is related to increased tumour aggressiveness as extrathyroid invasion, lymph node metastasis and higher grade staging [19], in accordance with the notion that EMT gene expression pattern is present in aggressive forms of PTC. Since periostin overexpression is a common event in epithelial tumours, knowledge of the mechanisms regulating transcription of periostin gene is demanding. Previous work has shown that periostin expression is regulated by Twist, a basic helix–loop–helix transcription factor, involved in cell proliferation, migration and differentiation [20]. Here, by taking the advantage of thyroid cancer cell line model, we investigated two major events having a role in the control of gene transcription: epigenetic marks of active transcription and effects of transcription factors. In discrepancy with current knowledge, we found no correlation between epigenetic marks of active transcription and expression of periostin gene. Instead, we found that the transcription factor p73 plays a role in the control of periostin expression.

2 Materials and methods

2.1 Cell lines and transfections

TPC-1 and BCPAP cell lines, derived from papillary thyroid carcinoma (PTC), WRO cells derived from human follicular thyroid carcinoma (FTC), FRO cells derived from human undifferentiated thyroid carcinoma (UC) [21], were grown in DMEM supplemented with 10% fetal bovine serum. HeLa cells were grown in DMEM supplemented with 10% calf serum.

The calcium phosphate co-precipitation method was used for transfections, as described elsewhere [22]. Briefly HeLa or TPC-1 cell lines were plated at 6 x 10^5 cell/100 mm culture dish, 20 h prior to transfection. The plasmid were used in the following amount (μg/plate): Periostin promoter construct, Periostin563-LUC, [20] 6 μg; pCDNA3.0-TAp73α, pCDNA3.0-TAp73β, pCDNA3.0-ΔNp73α [23] 2 μg; CMV-Pax8, 2 μg; CMV-Hex, 2 μg; CMV-TTF-1, 2 μg; CMV-FOXE1, 2 μg; CMV-FOXE1 Mut, 2 μg; RSV-CAT 2 μg.

Cells were harvested 48 h after transfection, and cell extracts were prepared by a standard freeze and thaw procedure. Luciferase (LUC) activity was measured by a chemiluminescence procedure [22]. The transfection efficiency was normalized by cotransfecting the RSV-CAT plasmid which contains the Raus Sarcoma Virus promoter linked to the chloramphenicol acetyltransferase (CAT) gene. CAT activity was measured by an ELISA method (Amersham).

To measure p73 effecs on periostin mRNA levels, TPC1 were stably transfected with TAp73α TAp73β ΔNp73 expression vectors.

2.2 Real time PCR

Quantitative PCR analysis of periostin mRNA expression was performed as previously described [24]. Briefly, total RNA from cell lines was extracted with RNeasy protect mini kit (Qiagen). One microgram of total RNA was reverse transcribed to single-strand cDNA using random exaprimers and 200 U MMLV reverse transcriptase (Invitrogen) in a final volume of 20 μl at 42°C for 50 min followed by heating at 70°C for 15 min. Real-time PCRs were performed using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

A 25 μl reaction mixture containing 5 μl cDNA template, 12.5 μl TaqMan Universal PCR master mix (Applied Biosystems), and 1.25 μl primer probe mixture was amplified using the following thermal cycler parameters: incubation at 50°C for 2 min and denaturation at 95°C for 10 min, then 40 cycles of the amplification step (denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min). The ΔCT method,

by means of the SDS software (Applied Biosystems), was used to calculate the mRNA levels. Oligonucleotide primers and probes for periostin were purchased from Applied Biosystems as Assays-on-Demand Gene Expression Products. Oligonucleotide, primers and probes for p73 are described in Table 1. Oligonucleotide primers and probes for the endogenous control β-glucuronidase are described by Beillard et al. [25].

2.3 Western blot

Material to be analyzed by Western Blot was obtained by acidic extraction. Briefly, cell pellet was resuspended with HCl 0.5 M additioned with PMSF and Protease Inhibitor Cocktail. Then the lisate was sonicated for 10 s three times and then centrifuged at 2,500 g, 10 min, 4°C. The supernatant was then collected and processed twice as described above. The total supernatant of the three extractions was precipitated with ten volumes of cold acetone, at -20°C over night. The sample was then centrifuged at 2,500 g, 15 min, 4°C, the supernatant was discarded and the pellet was resuspended in water. For Western blot analysis, 25 μg of protein extracts were electrophoresed on 12% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and these were saturated by incubating for 1 h with 5% non-fat dry milk in PBS/0.1% Tween 20. The membranes were then incubated overnight with the rabbit polyclonal anti-acetyl-histone H3 antibody (Upstate) or rabbit polyclonal anti H3 antibody (BioVisio). After three washes with PBS/0.1 Tween 20, membranes were incubated with anti-rabbit immunoglobulin coupled to 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 (BioRad) with the chemiluminescence procedure (Amersham Bioscence).

2.4 Chromatin immunoprecipitation assay (ChIP)

TPC-1, BCPAP, WRO and FRO cells (treated or not with TSA or SAHA) were cross-linked by the addition of formaldehyde to a final concentration of 1% for 10 min before harvesting. Plates were rinsed twice with ice cold PBS and cells were scraped off the plates, resuspended in cell lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1 and protease inhibitors) and sonicated to generate chromatin to an average length of about 200–600 bp. After centrifugation at 14,000 rpm for 10 min at 4°C, samples were diluted 10 fold with dilution buffer (0.01% SDS, 1.1% Triton x-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH 8.1, 167 mM NaCl). 10% of this was saved as total input of chromatin and was processed with the eluted immunoprecipitates beginning at the crosslink reversal step. The samples were precleared with 80 μl salmon sperm DNA/protein-A-Agarose beads for 30 min at 4°C. Agarose beads were pellet by centrifugation, the supernatant fraction was collected and immunoprecipitated overnight at 4°C with 10 μg anti-H3 acetylated monoclonal antibody (Upstate) or H3K4 trimethyl polyclonal rabbit (Active Motif). For a negative control, an immunoprecipitation with pre-immune serum was performed. Samples and negative controls were incubated with 60 μl of salmon sperm DNA/protein-A-Agarose beads for 1 h at 4°C. Agarose beads were pellet by centrifugation and washed for 5 min on a rotating platform with 1 ml of each buffer: Low salt, High salt, LiCl wash buffer, TE Buffer. Immunocomplexes were eluted with elution buffer (1% SDS, 50 mM $NaHCO_3$) and crosslinks were reverted by incubation at 65°C for 4 h. Samples were added with proteinase K, Tris HCl pH 6.5 and EDTA 0.5 M and incubated for 1 h at 45°C. DNA was purified with phenol/chloroform and used as template in a quantitative PCR to detect the presence of promoter or encoding sequences of Periostin, or GAPDH using the primers described in Tab. 1. After quantitative PCR reactions, the acetylated or methylated H3 levels were determined as ratio of signals recorded after and before (input) immunoprecipitation. Results of ChIP assays are expressed as fraction of the input.

2.5 Statistical analysis

Significance of difference among mean values was evaluated by the two-tail unpaired *t* test, using online software (http://www.graphpad.com).

Table 1 Oligonucleotide primers used in quantitative PCR reactions

	FORWARD	REVERSE	PROBE
Periostin promoter	5'-ATGAAAAGGAAAAGTAGCTCA ATGTG-3'	5'-GAAGCATCGGCAACTTCAAA TT-3'	5'-ACTAACCAAAACAAATCTTAG C-3'
Periostin encoding	5'-ATCCATGGGAACCAGATTGC-3'	5'-TTGTGTAAGCACACGGTCAAT G-3'	5'-ACAAATGGTGTTGTCCATG-3'
GAPDH	5'-CAAAGCTGGTGTGGGAGG-3'	5'-CTCCTGGAAGATGGTGATGG-3'	5'-CAAGCTTCCCGTTCTCAGCC-3'
TAp73	5'-GGAGGGCATGACTACATCTGTC A-3'	5'-GCGCGGCTGCTCATCT-3'	5'-CAGTTCAATCTGCTGAGCA-3'
ΔNp73	5'-GCGCGGCTGCTCATCT-3'	5'-GCGCGGCTGCTCATCT-3'	5'-CAGTTCAATCTGCTGAGCA-3'

3 Results

3.1 Relationship between periostin mRNA levels and post-transcriptional modifications of H3 histone levels

In order to explore mechanisms controlling periostin expression in thyroid cancer, four different thyroid tumour-derived cell lines were chosen: BCPAP and TPC-1 (from PTCs), WRO (from FTC), FRO (from UC). We have previously demonstrated that BCPAP, WRO and FRO cell lines express periostin mRNA, whereas the TPC-1 cell line does not exhibit this property [19]. Since histone acetylation at the level of gene promoters is associated to active transcription [26], we tested whether in thyroid cancer cells periostin gene expression is regulated by this mechanism. To this aim, a quantitative ChIP assay was performed with an antibody specifically directed against the acetylated form of H3K9 and H3K14, which have been reported to be involved in active transcription [27]. In these ChIP assays we investigated the H3 acetylated levels at the sequences of both promoter and coding region of periostin gene. In fact, in disagreement with H3 acetylation at the promoter level, H3 acetylation at the level of the encoding region may have a silencing effect [28]. Figure 1 shows that periostin mRNA levels were higher in WRO and FRO cells than in BC-PAP cells. In contrast, in TPC-1 cells periostin mRNA was undetectable. No correlation was found with acetylated H3 histone, both at the promoter and coding sequence level. In fact, in TPC-1 cells the levels of acetylated H3 were similar to those observed in WRO. In contrast, levels of acetylated H3 were undetectable, both at promoter and at coding sequence levels of periostin gene, in FRO cells.

In order to understand why the levels of acetylated H3 in FRO cells were undetectable, the ChIP assay was performed with the housekeeping gene GAPDH. Figure 2a shows that levels of acetylated H3 linked to GAPDH gene were very high in TPC-1 cells, intermediate in BCPAP and WRO cells (10 and 6-fold less than TPC-1, respectively) and very low in FRO cells (100-fold less than TPC1). Thus,

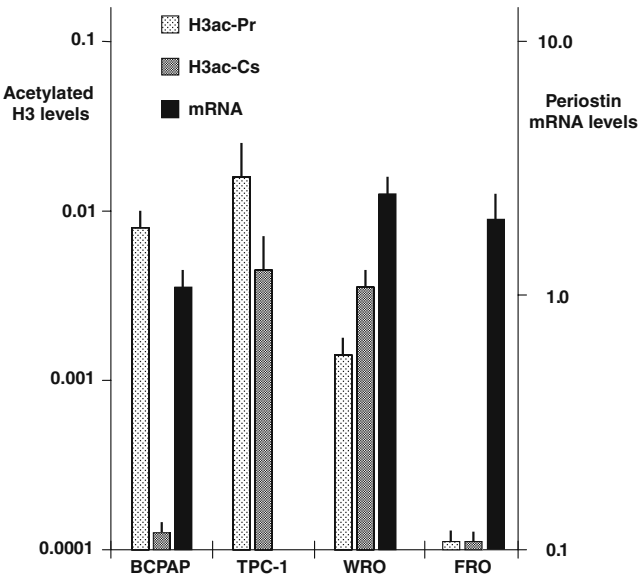

Fig. 1 H3 acetylation state of periostin gene and periostin mRNA levels in thyroid tumour cell lines. BCPAP, TPC-1, WRO and FRO cells were subjected to ChiP assays and quantitative RT-PCR to detect levels of acetylated H3 on either periostin promoter (H3ac-Pr, light gray bars) or periostin coding sequence (H3ac-Cs, dark gray bars) and periostin mRNA levels (black bars) as described in Materials and Methods. Each bar indicate the mean value±SEM of three determinations

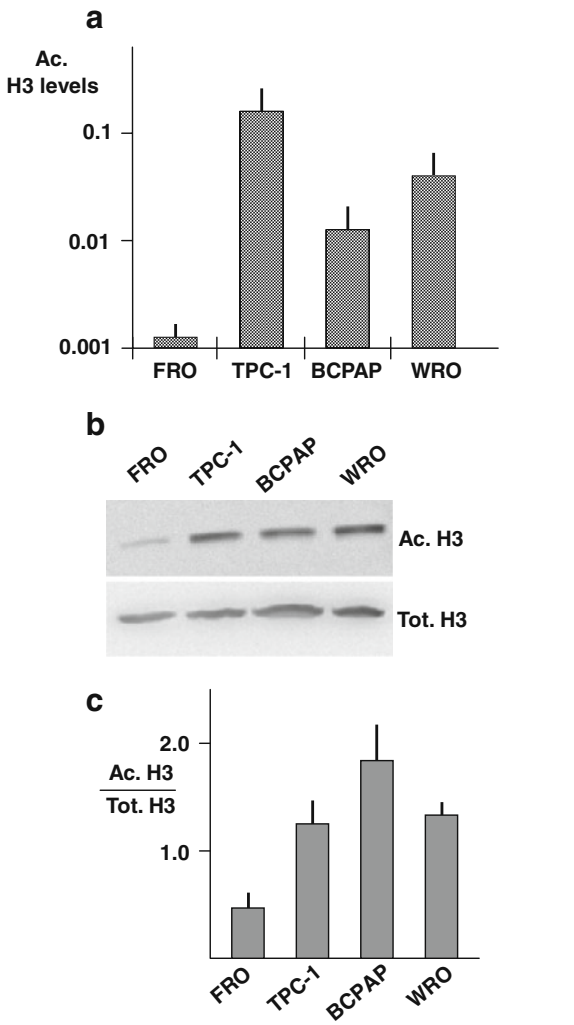

Fig. 2 GAPDH and global H3 acetylation state in thyroid tumour cell lines. **a** H3 acetylation state of the GAPDH gene in BCPAP, TPC-1, WRO and FRO cells. Each bar indicate the mean value±SEM of three determinations. Values of FRO cells are significantly different to values of other cell lines (always $p<001$). **b** Western blot image of total and acetylated H3 levels. **c** quantification of Western blots. Results are expressed as ratio between acetylated H3 and total H3; each bar indicate the mean value±SEM of three determinations. Values of FRO cells are significantly different to values of other cell lines (always $p<001$)

these results suggest that FRO cells has genomic lower levels of acetylated H3 in comparison with the other cell lines. In order to test this hypothesis, global levels of acetylated histone H3 were evaluated by western blot analysis. Indeed, Figs. 2b and c shows that global levels of acetylated H3 are reduced in FRO cells compared to BCPAP, TPC-1 and FRO cell lines.

Since the different steady state H3 histone acetylation levels among the cell lines tested may give rise to some adaptation mechanisms in the transcription process, we wanted to explore whether acute H3 acetylation increase may affect periostin gene transcription. It is known that HDAC inhibition may increase or decrease acetylated H3 levels of transcriptional regulatory sequences resulting in increase or decrease, respectively, of gene expression [29]. Therefore, to test the hypothesis of no relationship between acetylated H3 levels at the promoter and expression of periostin gene, cell lines showing highest periostin mRNA levels (WRO and FRO) were treated with HDAC inhibitors tricostatin A (TSA) or suberoylanilide hydroxamic acid (SAHA) (300 nM and 4 μM, respectively, for 24 h). Then, periostin mRNA and acetylated H3 levels at periostin promoter were measured. As shown in Fig. 3, in both cell lines either TSA or SAHA treatment significantly decreases periostin mRNA levels. However, both compounds increase acetylated H3 levels at periostin promoter (Fig. 3). Thus, these data indicate that levels of acetylated H3 at the promoter level do not play a major role in control of periostin gene expression.

In addition to acetylation, a different H3 histone post-transcriptional modification associated to active transcription is the trimethylation of lysine 4 (H3K4me3) [30, 31]. Thus, by using a specific antibody against H3K4me3, we tested whether TSA or SAHA treatment of WRO and FRO cells is able to modify this post-transcriptional modification. Results are shown in Fig. 4. The two cell lines show very similar basal levels of H3K4me3. However, while in WRO cells either TSA or SAHA significantly increases H3K4me3 levels, in FRO cells no significant modification was detected. We conclude, therefore, that histone marks of transcriptional activation (H3 acetylation at lysines 9 and 14 and trimethylation at lysine 4) at the level of periostin promoter do not correlate with levels of periostin gene expression.

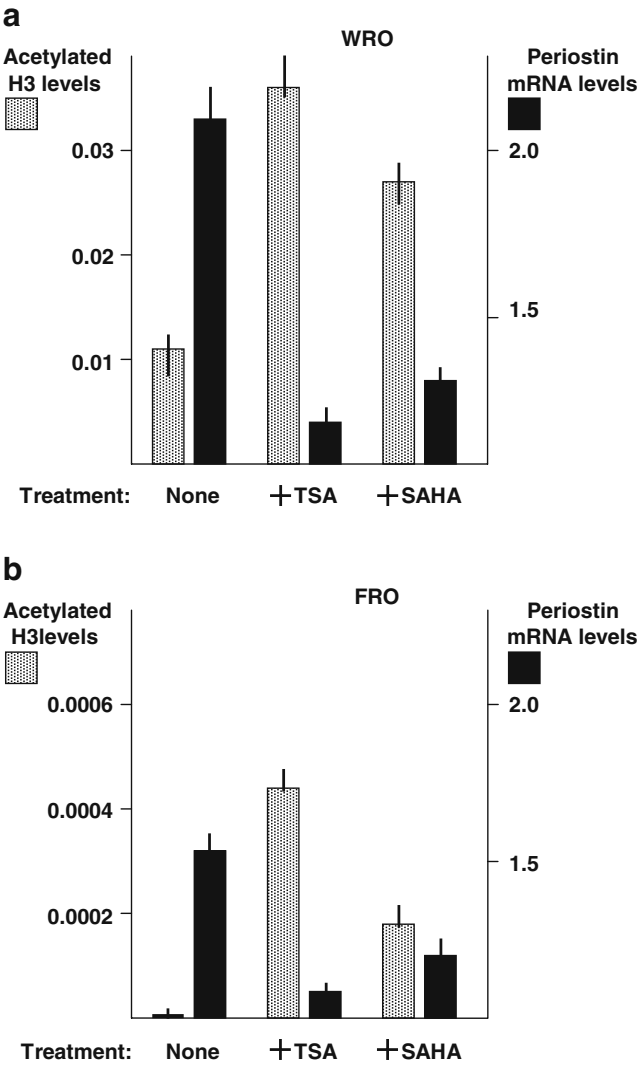

Fig. 3 Effect of TSA or SAHA on H3 acetylation state of periostin gene promoter and periostin mRNA levels in WRO and FRO cells. Cell lines were treated for 24 h with TSA or SAHA (300 nM and 4 μM, respectively) and then subjected to ChiP assays and quantitative RT-PCR to detect levels of acetylated H3 on periostin promoter (H3ac-Pr, light gray bars) and periostin mRNA levels (black bars). **a** WRO cells; **b** FRO cells. In both panels, TSA and SAHA induce significant mRNA levels decrease and H3 acetylation increase (for either mRNA levels or H3 acetylation: $p<0.01$ compared to untreated cells). Each bar indicates the mean value±SEM of three determinations

3.2 Effects of transcription factors on periostin promoter

To identify the possible mechanisms responsible for the increase of periostin expression occurring in thyroid malignancies, a different hypothesis was tested. As the periostin promoter has been identified [20] and, a correlation between the activity of periostin promoter (assessed by reporter assays) and expression of periostin gene has been already shown in thyroid cancer cells [19], we investigated effects of transcriptional activator/repressors on the activity of periostin promoter. Several different thyroid-specific transcription factors were evaluated: PAX8, HEX, TTF-1, FOXE1, which are expressed in normal thyroid cells [32]. Since expression or function of these thyroid-specific transcription factors is often down-regulated in thyroid carcinomas [33], it is possible that some of these factors may exert repression on periostin promoter. In contrast, several transcription factors are aberrantly expressed in thyroid cancer cells including the p53 paralog p73. [34].

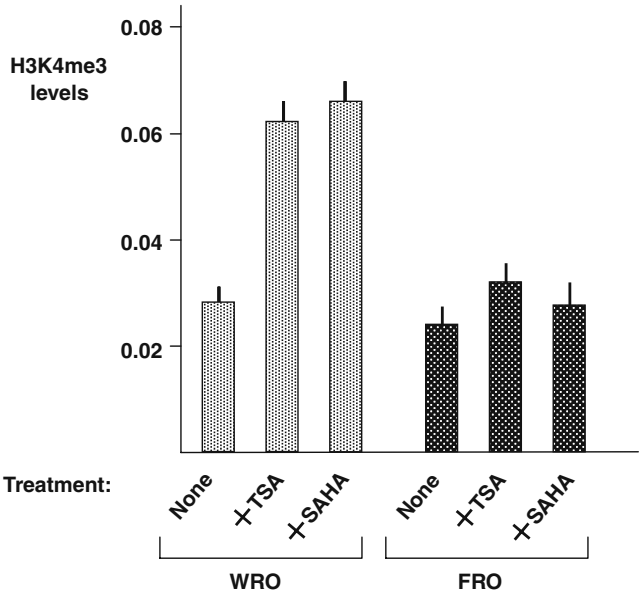

Fig. 4 Effect of TSA or SAHA on H3K4me3 levels of periostin promoter in WRO and FRO cells. Cell lines were treated for 24 h with TSA or SAHA (300 nM and 4 μM, respectively) and then subjected to ChiP assays to detect H3K4me3 levels on periostin promoter. Each bar indicates the mean value±SEM of three determinations. In WRO cells treated with either TSA or SAHA, H3K4me3 levels are significantly higher with respect to untreated cells ($p<0.01$ in either condition)

Therefore, p73 could activate periostin promoter. To test these hypotheses HeLa cells were transfected with luciferase-conjugated promoter along with either thyroid specific transcription factors or p73 isoforms. Figure 5a shows that among thyroid-specific transcripton factors, PAX8 and TTF-1 have no effects and HEX exerts a slight inhibitory action. FOXE1, instead, induces a 2-fold increase of periostin promoter activity. A FOXE1 mutant, lacking the domain responsible for the transcriptional functions of this factor [35], is not longer able to activate periostin promoter. Figure 5b shows the results obtained with p73 isoforms TAp73α, TAp73β and ΔNp73: only ΔNp73 has a strong activating effect, while TAp73α has no effect and TAp73β a slight inhibitory effect.

In order to confirm that p73 may be responsible for the overexpression of periostin in thyroid carcinomas, two different approaches were used. First, the relationship between the expression of periostin and p73 isoforms was evaluated in BCPAP, TPC-1, WRO and, FRO cell lines. Figure 6a shows the quantitative-RT PCR data indicating that in TPC-1 cells the levels of periostin gene expression are undetectable as well as the levels of TA and ΔNp73 isoform are extremely low. Instead, the remaining cell lines, which express periostin, display relatively high levels of ΔNp73 mRNA. If a positive correlation between ΔNp73 and periostin expression may exist, it is predictable that forced expression of ΔNp73 in TPC-1 cells should be able to increases periostin mRNA levels. Thus, TPC-1 cells were stably transfected with expression vectors for TA-p73α, TAp73β and ΔNp73 proteins and levels of periostin mRNA evaluated by quantitative RT-PCR. Figure 6b shows that, expression of TAp73α and TAp73β induced only slight increase and decrease of periostin mRNA levels, respectively. Differently, expression of ΔNp73 induced a significant increase of periostin mRNA levels (4-fold, $p<0.05$). These results were in accordance with those obtained in HeLa cells transfected with periostin promoter and further confirm the positive relationship between periostin and ΔNp73.

In the light of these results we tested whether the effects of HDAC inhibitors on periostin mRNA levels are mediated by ΔNp73. To this end, TSA and SAHA effect on ΔNp73 mRNA levels were investigated in FRO and WRO cells. These cells were treated for 24 h with TSA or SAHA (300 nM and 4 μM, respectively), and mRNA levels of ΔNp73 measured by quantitative RT-PCR. Figure 7 shows that in FRO cells both TSA and SAHA induce significant decrease of ΔNp73 mRNA levels, while in WRO cells, no significant modification was detected. Thus, effects of HDAC inhibitors on ΔNp73 mRNA levels are cell line-dependent. In particular, reduction of ΔNp73 in FRO cells could explain the effect of HDAC inhibitors on periostin mRNA levels.

4 Discussion

Several lines of evidence indicate that periostin expression in thyroid cancer is associated to a more aggressive phenotype [19, 36]. Therefore, mechanisms regulating periostin expression in thyroid carcinomas may be relevant to the identification of molecular pathways responsible for tumour progression. Our previous investigation indicates that periostin overexpression in PTCs might be, at least in part, due to transcriptional mechanisms [19]. Thus, we explored two major potential mechanisms involved in the regulation of periostin gene transcription: a) histone post-translational modifications, which play a major role in control of chromatin structure and, b) transcription factors, which have an essential role in the assembly and activity of transcriptional machinery. No relationship was observed between the H3 acetylation status at levels of either periostin promoter or coding sequence and periostin expression. In particular, TPC-1 cells show high levels of acetylated H3 however, no periostin expression. Contrarily, FRO cells shows high periostin mRNA levels despite extremely low levels of acetylated H3. These results are unexpected since histone acetylation, particularly at the promoter level, is believed to favour gene transcription [26]. However, our ChIP experiments, using an antibody

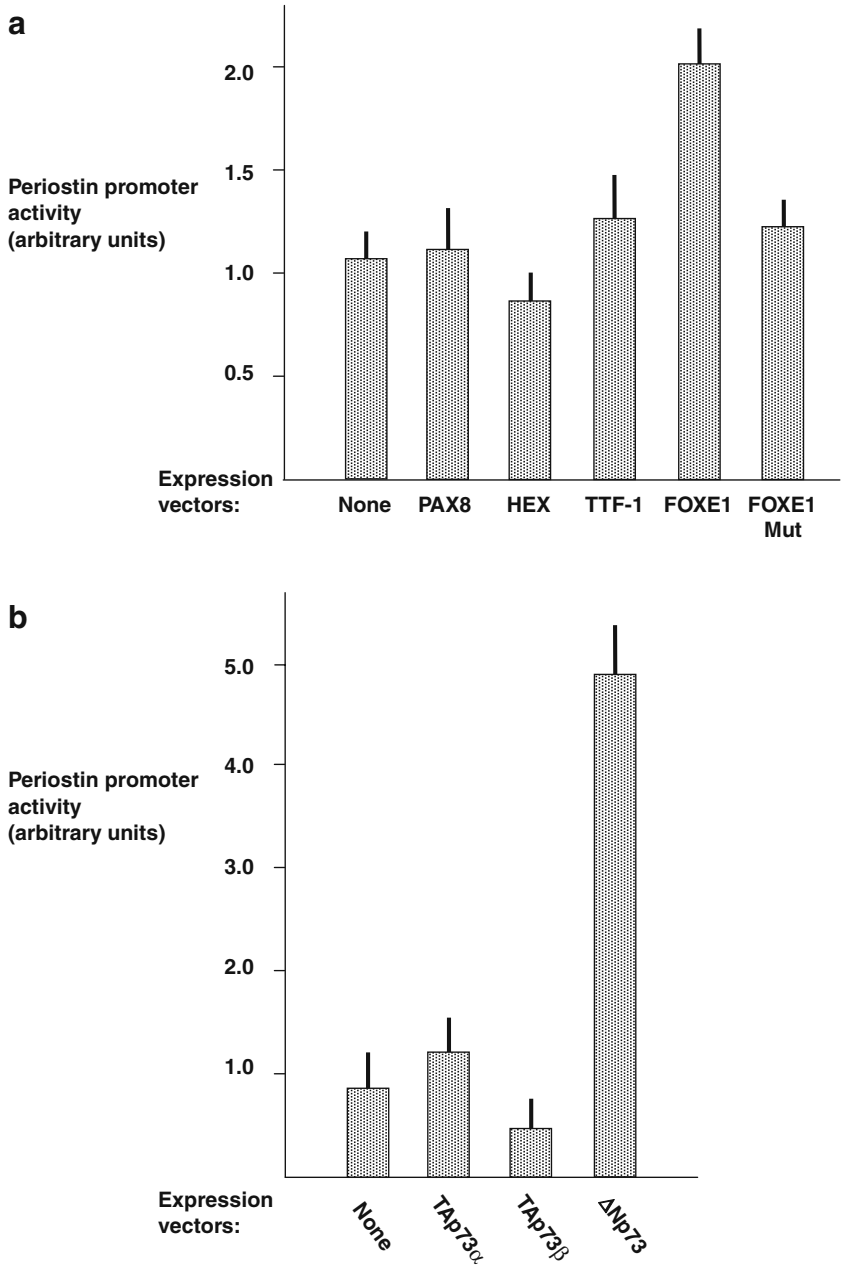

Fig. 5 Effects of different transcription factors on periostin promoter activity. HeLa cells were transfected with the periostin promoter construct and without/with expression vectors for PAX8, HEX, TTF-1 and FOXE1 and three isoforms of p73, as described in Materials and Methods. **a** effect of PAX8, HEX, TTF-1 and FOXE1. The difference between the absence of expression vector (None) and FOXE1 is the only significant ($p<0.05$). **b** effect of TAp73α, TAp73β and ΔNp73. The difference between the absence of expression vector (None) and ΔNp73 is the only significant ($p<0.01$). In both panels each bar indicates the mean value ±SEM of three determinations

against the acetylated forms of lysines at position 9 and 14 of H3 histone, have been unsuccessful in establishing any correlation between periostin mRNA levels and acetylation of histone H3 residues at level of periostin promoter.

Indeed, treatment of WRO and FRO cells with the HDAC inhibitors TSA or SAHA increases levels of acetylated H3, however it decreases periostin mRNA levels. Since data obtained with two different compounds (TSA and SAHA) are very similar, we conclude that decrease of periostin mRNA levels can be considered a "bona fide" effect of HDAC inhibition. These results are not in agreement with those recently reported by Rada-Inglesias et al., using the HDAC inhibitor sodium butyrate. These authors, in fact, observed association between reduction of gene expression and histone deacetylation [29]. In WRO and FRO cells, reduction of periostin expression occurs when also sodium butyrate is used as HDAC inhibitor (data not shown).

In addition to H3 acetylation, the modification of H3K4me3 upon TSA or SAHA treatment was investigated. Both TSA and SAHA increase H3K4me3 levels in WRO cells; in FRO cells, instead, neither drug induce significant modifications. It is known that HDAC inhibitors may increase H3K4me3 levels [37]. However, contrarily to what we observed on periostin promoter, this effect has been always associated to increase of gene expression [38, 39].

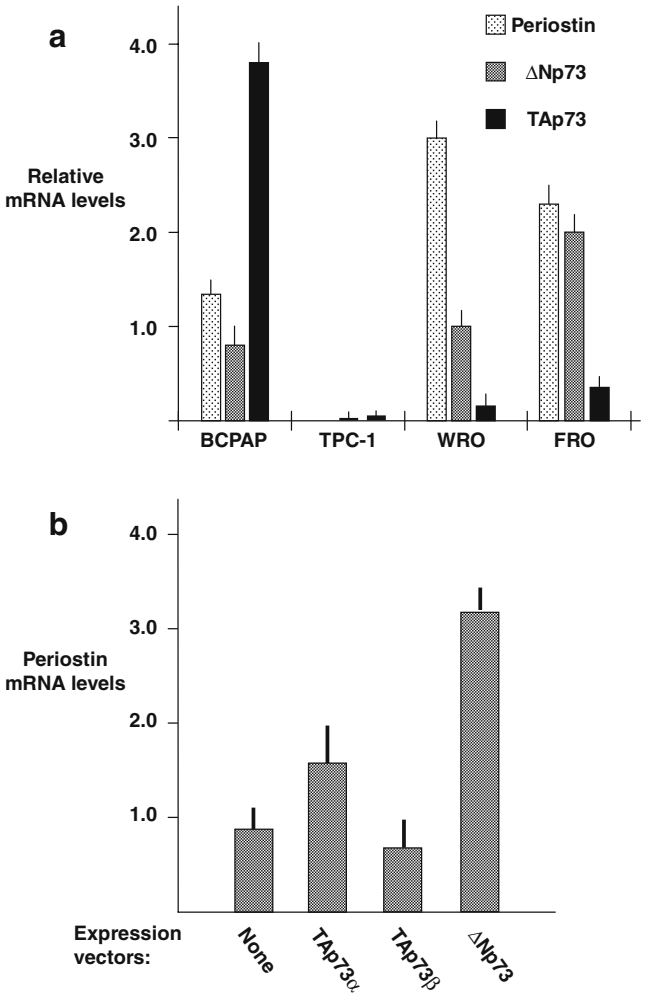

Fig. 6 Expression of p73 isoforms in thyroid tumour cell lines and effects on periostin mRNA levels. **a** periostin and p73 isoforms expression in thyroid tumour cells. Periostin, ΔNp73 and TAp73 mRNA levels in BCPAP, TPC-1, WRO and FRO cells were evaluated by quantitative RT-PCR as described in Materials and Methods. **b** effect of p73 isoforms on periostin mRNA levels. TPC-1 cells were stably transfected with the expression vectors for TAp73α, TAp73β and ΔNp73 isoforms as described in Materials and Methods. After total RNA extraction, periostin mRNA were evaluated by quantitative RT-PCR. The difference between the absence of expression vector (None) and ΔNp73 is the only significant ($p<0.01$). In both panels each bar indicates the mean value±SEM of three determinations

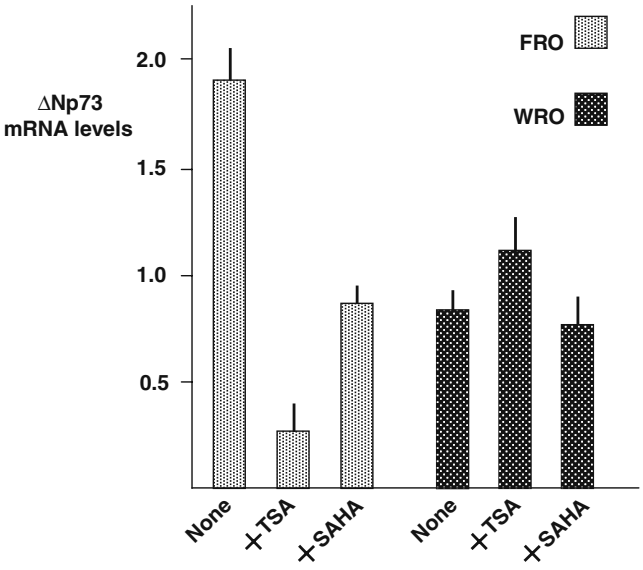

Fig. 7 Effect of TSA and SAHA on ΔNp73 mRNA levels. FRO and WRO cell lines were treated with either TSA or SAHA (300 nM and 4 μM, respectively) for 24 h. Then, total RNA was extracted and ΔNp73 mRNA levels were evaluated as described in Materials and Methods. Each bar indicates the mean value±SEM of three determinations. For FRO cells either TSA or SAHA-treated cells shows significant difference compared to untreated cells (None) ($p<0.01$). For WRO cells either TSA or SAHA-treated cells shows no significant difference compared to untreated cells (None)

In thyroid cancer cell lines the status of H3K4me3 does not explain the decrease of periostin mRNA levels upon treatment with HDAC inhibitors. Altogether, our results suggest that in thyroid cancer cells periostin gene expression is regulated by mechanisms that are independent from several H3 post-translational modification at the level of periostin promoter. In particular, no correlation exists between major epigenetic marks of active transcription and periostin gene expression. Effects of HDAC inhibitors on WRO cells are particularly instructive. In this cell line, in fact, increase of major epigenetic marks of transcriptional activation is associated to decrease of periostin mRNA levels. A simple model to explain our results is that in thyroid cancer cell lines the chromatin structure at the level of periostin gene is in an open configuration, therein activity of periostin promoter is controlled by transcription factors able to activate or repress the transcriptional machinery. With regards to transcription factors, we show that ΔNp73 is able to activate periostin promoter. Moreover, expression of this protein in TPC-1 cells activates expression of the endogenous periostin gene. Coherently, in thyroid tumour cell lines we show a correlation between the presence of periostin and ΔNp73 mRNAs. Together with data indicating no correlation between periostin expression and acetylated H3 levels, we suggest that effects of ΔNp73 on periostin promoter are not dependent from H3 histone acetylation. This notion is not completely unexpected; it has been shown, essentially, that, although the histone acetylating protein p300 is a coactivator of p73, its acetylase activity is not required to stimulate the p73-dependent transcription [40].

Treatment with TSA and SAHA reduced ΔNp73 mRNA levels in FRO cells, but not in WRO cell line. Therefore, effects of HDAC inhibitors on ΔNp73 are cell line-dependent. In fact, it has been reported that treatment of HeLa cells with sodium butyrate increases ΔNp73 mRNA levels [41]. Since HDAC inhibition caused no effect on ΔNp73 mRNA levels in WRO cells, only in FRO cells effect of HDAC inhibitors on periostin gene expression

could occur via modification of ΔNp73 expression. This heterogeneous behaviour indicate the complexity of periostin gene expression regulation.

It is interesting to note that ΔNp73, but not TAp73 is able to significantly increase periostin gene transcription. This observation, merged with the absence of periostin expression in TPC-1 cells, suggests that p53 may be a repressor of periostin gene. TPC-1 cells express wtp53, while cell lines positive for periostin expression like BCPAP, WRO and FRO are p53 null [34]. Since ΔNp73 may act as a p53 antagonist in several cell systems [42], it is possible that ΔNp73 stimulates periostin gene transcription in TPC-1 cells by removing the p53 transcriptional inhibition. To address this issue, the effect of ectopic expression of ΔNp73, should be evaluated in thyroid cancer cells with different p53 genetic background. However, a non-p53-dependent effect of ΔNp73 on periostin promoter cannot be excluded. Previous reports have shown that ΔNp73 may selectively modulate gene expression in thyroid cancer cells. Infact, in this cell model, ΔNp73 is able to repress PTEN promoter in a p53-independent manner [23]. From this perspective, periostin induction by ΔNp73 may evolve a mechanism of thyroid cancer progression via ΔNp73. Several data indicate that the p53 protein family is involved in control of expression of genes involved in EMT. In particular, it has been shown that p53 exerts repression of molecular modifications that are typical of EMT, such as E-cadherin down-regulation and SNAIL and SLUG overexpression [43–45]. Since ΔNp73 may act as a p53 antagonist, this p73 isoform likely contributes to EMT in thyroid cancer.

Among thyroid-specific transcription factors PAX8, HEX and TTF-1 have no effects on periostin promoter. Only FOXE1, induces significant activation of this promoter, suggesting that this protein may contribute to periostin gene expression in thyroid tumours. Accordingly, FOXE1 expression spans a large fraction of thyroid malignancies [46, 47]. Additionally, Sequeira et al. demonstrated that an increased FOXE1 expression paralleled the dedifferentiation process of thyroid carcinomas [46]. Interestingly, based upon genetic analysis of thyroid carcinomas, it has been hypothesised that increased FOXE1 expression could be related to a motile advantage of malignant thyroid cells [48]. Moreover, genes encoding FOX proteins have been recently identified as contributing the molecular signature for EMT in a human colon cancer [49]. Thus, detailed investigations on EMT mechanism in thyroid cancer could have impact to improve management of this disease.

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