Translational Thyroidology / Original Paper

European
Thyroid Journal

Eur Thyroid J 2015;4:213–221
DOI: 10.1159/000441355

Received: May 11, 2015
Accepted after revision: September 29, 2015
Published online: November 21, 2015

miR-130b-3p Upregulation Contributes to the Development of Thyroid Adenomas Targeting *CCDC6* Gene

Vincenza Leone[a] Concetta Langella[a] Francesco Esposito[a] Marco De Martino[a]
Myriam Decaussin-Petrucci[d] Gennaro Chiappetta[b] Antonio Bianco[c]
Alfredo Fusco[a, e]

[a]Istituto di Endocrinologia ed Oncologia Sperimentale-CNR, Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Scuola di Medicina e Chirurgia di Napoli, Università degli Studi di Napoli 'Federico II', [b]Istituto Nazionale per lo Studio e la Cura dei Tumori 'Fondazione Giovanni Pascale', IRCCS, and [c]Dipartimento di Sanità Pubblica, Università di Napoli Federico II, Naples, Italy; [d]Department of Pathology, Lyon Sud Hospital Center, Hospices Civils de Lyon, Lyon, France; [e]Instituto Nacional de Cancer (INCA), Rio de Janeiro, Brazil

Key Words
MicroRNA · Thyroid · Adenoma · Cell cycle

Abstract
We have previously studied the function of microRNAs (miRNAs) in thyroid cells using the differentiated rat thyroid PC Cl 3 cells that need thyrotropin (TSH) for their growth. The miRNA expression profile examination allowed the detection of a set of miRNAs downregulated and upregulated by TSH. Here, we first demonstrated that upregulation of miR-130b-3p occurs through a protein kinase A-cAMP-responsive element binding protein (CREB)-dependent mechanism. Then, we analyzed its expression in human thyroid follicular adenomas, where a constitutive CREB activation is frequently present. miR-130b-3p results in upregulation with a high fold-change in most thyroid follicular adenomas. Then, we identified *CCDC6*, coding for a protein that interacts with CREB1 leading to the transcriptional repression of CREB1 target genes, as a target of this miRNA. The targeting of CCDC6 by miR-130b-3p likely accounts for the mechanism by which its upregulation contributes to the development of thyroid adenomas increasing CREB1 activity.

Introduction

MicroRNAs (miRNAs) are small noncoding RNA molecules of 19–22 nucleotides in length that are capable of regulating gene expression at a translational level and binding to a complementary sequence found in the 3′-untranslated region (UTR) of target mRNA [1, 2]. They show a variety of crucial regulatory functions related to cell growth, development, differentiation and apoptosis [3–9].

We have previously analyzed the role of miRNAs in thyroid cell proliferation using the differentiated rat thyroid PC Cl 3 cells that require thyrotropin (TSH) for their growth. The analysis of the miRNA expression profile in

E-Mail karger@karger.com
www.karger.com/etj

Alfredo Fusco
Istituto di Endocrinologia ed Oncologia Sperimentale-CNR
Via Pansini 5
IT–80131 Naples (Italy)
E-Mail alfusco@unina.it

Table 1. miRNAs differentially expressed between PC Cl 3 treated with TSH and PC Cl 3 treated with BSA

Unique ID	Ratio (treatment TSH vs. BSA 30 min)
mmu miR 699	2.026
mmu miR 123	2.034
hsa miR 196a-1	2.038
hsa miR 198	2.086
hsa miR 213	2.119
hsa miR 103-1	2.164
mmu miR 302d	2.229
hsa miR 421	2.232
mmu miR 128b	2.258
mmu miR 696	2.293
hsa miR 181b-1	2.308
mmu miR 181b-1	2.338
mmu miR 323	2.352
hsa miR 26a	2.376
hsa miR 26b	2.398
mmu miR 361	2.43
hsa miR 339	2.504
hsa let-7d-v2	2.582
hsa miR 99a	2.653
hsa miR 361	2.712
mmu miR 342	2.87
hsa miR 342	2.871
mmu miR 130b	3.125
mmu miR 685	3.235
hsa miR 189	3.562
mmu miR 682	4.649
hsa miR 23a	5.451
hsa miR 487b	7.025

TSH-stimulated PC Cl 3 cells allowed the identification of a set of miRNAs downregulated and upregulated by TSH. Moreover, functional studies demonstrated that the positive and negative regulation of these miRNAs expression was critical for the TSH stimulatory effect [10, 11].

In this study we focus on miR-130b-3p, which resulted in upregulation by TSH in PC Cl 3 cells (table 1). First, we showed that the upregulation of this miRNA is protein kinase A-cAMP-responsive element binding protein (CREB) dependent, and that CREB1 binds the sequence upstream of the miRNA 130b-3p gene. These results prompted us to investigate the role of miR-130b-3p in cell proliferation associated with thyroid disorders due to constitutive CREB activation such as follicular adenomas (FTAs). We report that miR-130b-3p is drastically upregulated in the large majority of benign thyroid neoplasias such as FTAs, known to be frequently CREB dependent for proliferation, in comparison with normal thyroid. Interestingly, this miRNA has as a target the *CCDC6* gene that our previous studies have demonstrated to code for a protein able to interact with CREB1, leading to the transcriptional repression of CREB1 target genes [12].

Materials and Methods

Cell Lines and Transfection

Human embryonic kidney HEK-293 cells were cultured in DMEM containing 10% FBS. PC Cl 3 and FRTL5 were grown in Ham's F-12 medium, Coon's modification (Sigma-Aldrich, Milan, Italy) supplemented with 5% calf serum (Life Technologies Inc., Paisley, Pa., USA) in the presence of a mix containing six growth factors (6H: 10 nM TSH, 10 nM hydrocortisone, 100 nM insulin, 5 μg/ml transferrin, 5 nM somatostatin and 20 μg/ml glycyl-histidyl-lysine). The TSH used for the experiments was of bovine derivation purchased from Sigma-Aldrich, Milan, Italy. Transfections were performed as described previously [10, 11]. The transfection efficiency was about 85%, evaluated by transfection of AllStars (Sigma, Neg. siRNA AlexaFluor 488; Qiagen). Forskolin was obtained from Sigma (St. Louis, Mo., USA).

Authentication of Cell Lines

Human embryonic kidney HEK-293 cells were distributed by American Type Culture Collection (ATCC). The STR profile was carried out in the cell line HEK-293. The analysis of the STR profile revealed that the HEK-293 cell line does not show any difference when compared to the profiles published by one or more of the international databases. The passage number of cell lines used for the experiments was 25.

Rat PC Cl 3 and FRTL5 cells have been described elsewhere [13, 14]. The passage number of cell lines used for the experiments was 28.

Human Thyroid Tissue Samples

Normal and adenoma thyroid tissues were collected at the Service d'Anatomo-Pathologie, Centre Hospitalier Lyon Sud, France. The samples are included in the Biobank of the Lyon Sud Hospital, included in the 'Centre de Ressources Biologiques des Hospices Civils de Lyon'. Informed consent for the scientific use of biological material was obtained from the patients.

RNA Extraction and Real-Time Quantitative PCR

Total RNA was isolated from cells and human tissues with Trizol (Invitrogen) according to the manufacturer's instructions. Real-time quantitative PCR (qRT-PCR) for mature miRNA was carried out according to the manufacturer's instructions for the miScript System Kits (Qiagen). Reactions contained miScript Primer Sets (Qiagen), specific for miR-130b-3p and U6 (used to normalize RNA levels). qRT-PCR analysis for *CCDC6* and *AREG* were performed as previously described [15]. Primers for glucose-6-phosphate dehydrogenase *(G6PD)* were used for mRNA normalization. Each reaction was carried out in triplicate. To calculate the relative expression levels, we used the $2^{-\Delta\Delta CT}$ method [16]. Primer sequences were: CCDC6 F: GAGCTCTCCCGGAAACTGAT, CCDC6 R: CATCAGTTTGTTGACCTGGAAC; AREG F: GGTGAATGCAGATACATCGAGA, AREG R: CGTTCGCCAAAGTAATCCTG, and G6PD: TCCTCTATGTGGAGAATGAACG, G6PD: TCATTCAGAGCTTTGCCACA.

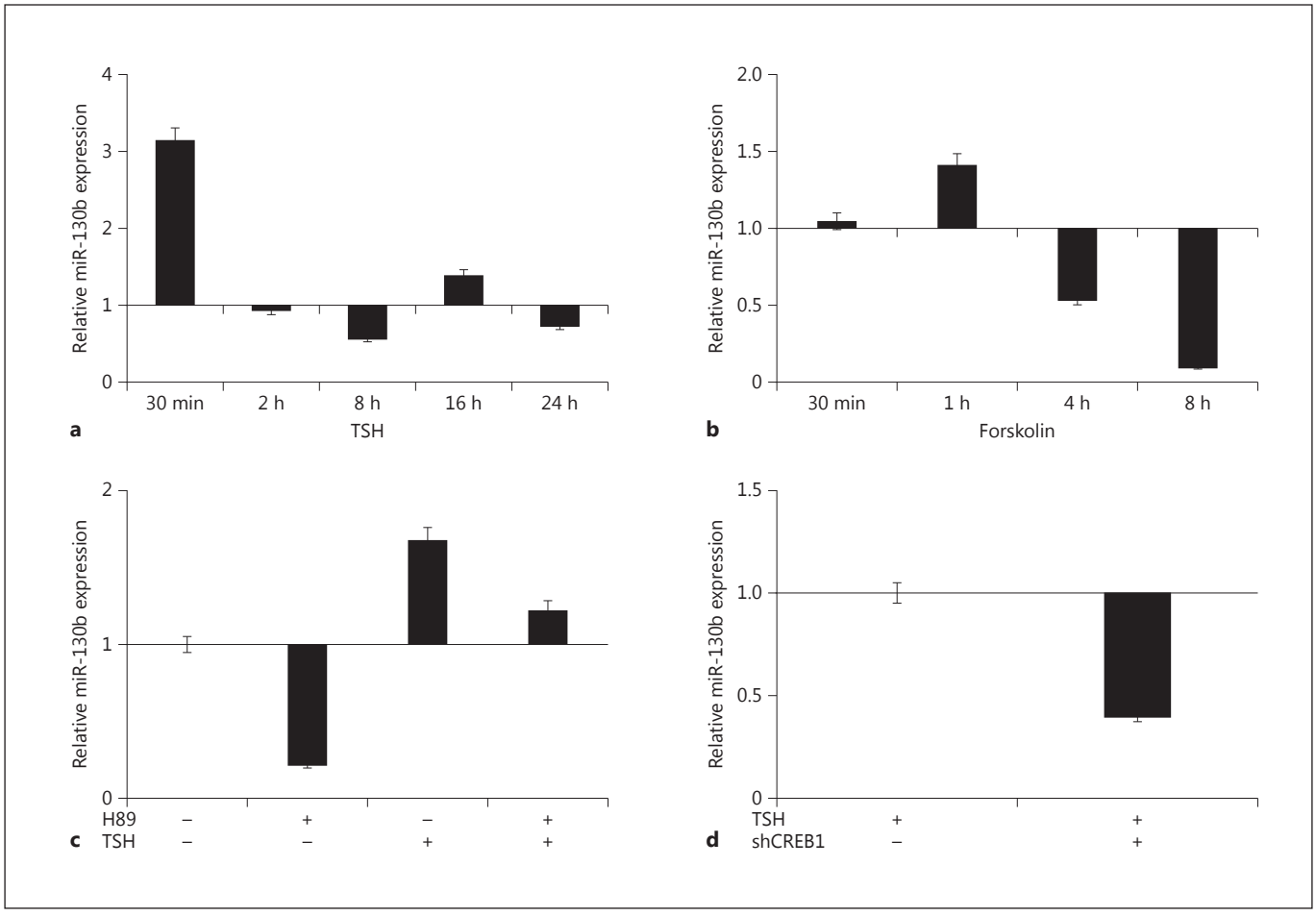

Fig. 1. miR-130b-3p is upregulated by TSH via a kinase A/CREB1 pathway. **a** Validation of miRNA microarray data by qRT-PCR. RNAs were extracted from PC Cl 3 cells treated with TSH (10 nM) at the indicated times. The relative expression values indicate the relative change in the expression levels between PC Cl 3 cells treated with TSH and PC Cl 3 cells treated with BSA, assuming that the value of the PC Cl 3 cells treated with BSA was equal to 1. U6 was used to normalize RNA levels. Each bar represents the mean value ± SE from three independent experiments performed in triplicate. **b** qRT-PCR analysis of miR-130b-3p was carried out on PC Cl 3 cells treated with forskolin (10 μM) at the indicated times. The relative expression values indicate the relative change in the expression levels between PC Cl 3 cells treated with forskolin and PC Cl 3 cells treated with DMSO, assuming that the value of the PC Cl 3 cells treated with DMSO was equal to 1. Each bar represents the mean value ± SE from three independent experiments performed in triplicate. **c** qRT-PCR analysis of miR-130b-3p was carried out on PC Cl 3 cells treated for 30 min with TSH or TSH plus H89 or H89 alone at 10 μM. Each bar represents the mean value ± SE from three independent experiments performed in triplicate. **d** qRT-PCR analysis of miR-130b-3p was carried out on PC Cl 3 cells treated for 30 min with TSH in the presence of shRNAs for *CREB1* or the scrambled control. The treatment with shRNAs for *CREB1* lasted 48 h. The relative expression values indicate the relative change in the expression levels between PC Cl 3 cells in the presence of shRNA or the scrambled control for *CREB1* and treated with TSH, assuming that the value of the PC Cl 3 cells treated with TSH was equal to 1.

Bioinformatic Prediction of miRNA Target Genes

Genes potentially targeted by the selected miRNAs were identified by using different online available tools such as TargetScan (www.targetscan.org), miRanda (www.microrna.org) or miRwalk (www.umm.uni-heidelberg.de/apps/zmf/mirwalk). For more details see the online supplemental information (for all online suppl. material, see online Supplementary Materials).

Chromatin Immunoprecipitation Assay

Chromatin samples were processed for ChIP experiments as reported elsewhere [12]. Samples were subjected to immunoprecipitation with the specific CREB1 antibody (Upstate Biotechnology). To calculate the percent of total chromatin, we used the $2^{-\Delta\Delta CT}$ method [16]. The sequences of the primers we used were: CRE-MIR-130 F: GCAGACCTGGATCTTCCACT, and CRE-MIR-130 R: GGCCTGGAGAAGCAGAACTA.

Western Blotting and Antibodies

Western blot analyses were performed as described previously [10, 11]. The blots were incubated with antibodies against CCDC6 [17], and after stripping with vinculin (sc-7649; Santa Cruz).

Luciferase Target Assays

They were performed as described previously [10, 11].

XTT Assay

It was performed as described previously [10, 11].

Flow Cytometry

PC Cl 3 cells were plated and synchronized by serum deprivation for 48 h. The cells were then transfected with 50 nmol/ml of pre-miR miRNA precursor or scrambled oligonucleotide using siPORT neoFX and collected after 72 h. After this, we proceeded as described previously [10, 11].

Statistical Analysis

Student's t test was used to determine the differences between the two population samples. Data are presented as means ± SE, and $p < 0.05$ was accepted as statistically significant.

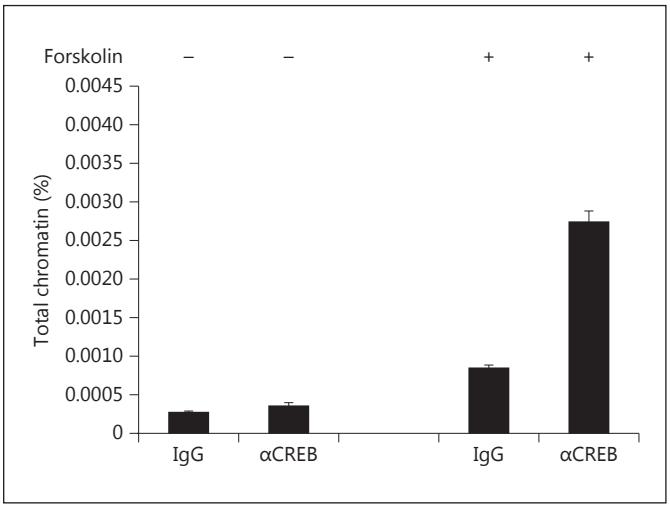

Fig. 2. CREB1 binds to the promoter of the miR-130b-3p gene. ChIP assay was carried out on PC Cl 3 cells treated with forskolin (10 μM) for 1 h. The precipitated DNA for CREB1 antibodies was used as a template for qRT-PCR with primers that amplify the upstream region of miRNA containing the CRE element. The percent of chromatin indicates the change in the immunoprecipitated chromatin by CREB1 antibody with respect to IgG used as control.

Results

miR-130b-3p Is Upregulated by TSH through a Protein Kinase A-CREB-Dependent Mechanism

In this study, we focused on the miR-130b-3p that was among the miRNAs upregulated by TSH (table 1) [10, 11]. First, qRT-PCR data confirmed the upregulation of the miR-130b-3p in PC Cl 3 cells 30 min after TSH treatment (fig. 1a), whereas at 2 h it was expressed at a level comparable to that of the control PC Cl 3 cells treated with BSA. Similar results have also been obtained with FRTL5 cells treated for 1, 4 and 8 h with TSH (online suppl. fig. 1). Indeed, there is a clear increase of miR-130b-3p expression at 1 h, whereas at 4 h the expression drastically decreases and at 8 h goes back to the levels of the control BSA-treated cells.

Next, we treated PC Cl 3 cells with forskolin (10 μM), which is a compound that increases the intracellular cAMP level, thus activating cAMP-dependent protein kinase and other cAMP receptor proteins and thereby mimicking the effects of TSH. Forskolin treatment resulted in an increased expression of miRNA 1 h after exposure to forskolin (fig. 1b), of which the levels were even lower at 4 h and 8 h compared with the control cells. It is worth noting that the times of miR-130b-3p induction by TSH and forskolin in PC Cl 3 are quite different, but this is not surprising since these compounds are not completely overlapping in their functions.

To determine whether the TSH-induced upregulation of miR-130b-3p is kinase A-dependent, we measured its expression after treatment for 30 min with TSH and the kinase A inhibitor H89 at a concentration of 10 μM in comparison with the PC Cl 3 cells treated with BSA and H89 or TSH alone. As shown in figure 1c, treatment with H89 plus TSH led to the downregulation of miR-130b-3p versus cells treated with TSH alone. Interestingly, the treatment of H89 alone leads to a reduced miR-130b-3p expression that likely depends on the basal endogenous kinase A activity that is inhibited by H89.

Next, we asked whether the upregulation of miR-130b-3p was dependent on the activation of the CREB1 transcription factor. Therefore, the PC Cl 3 cells were treated with shRNAs for *CREB1* for 48 h before TSH stimulation and we measured the expression of miR-130b-3p in PC Cl 3 cells treated with TSH (30 min) in the presence of shRNAs for *CREB1* or scrambled oligonucleotides. As shown in figure 1d, miR-130b-3p was downregulated in PC Cl 3 cells in which shRNAs significantly reduced CREB1 protein levels.

CREB1 Protein Directly Binds the miRNA Region Located Upstream of the miR-130b-3p Sequence

To confirm the molecular mechanisms underlying the upregulation of miRNA expression by the TSH-CREB

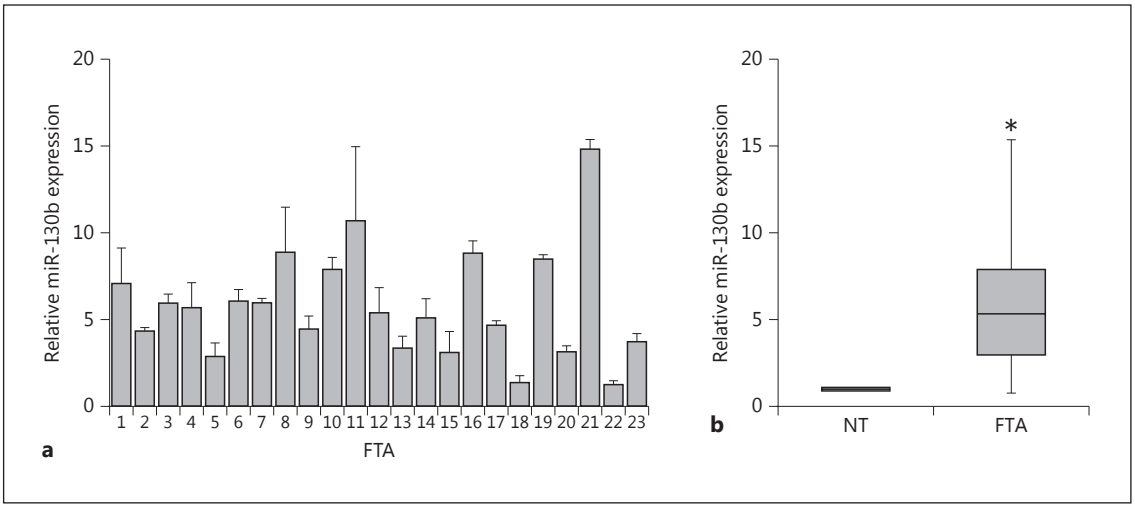

Fig. 3. Analysis of miR-130b-3p expression in FTAs by qRT-PCR. **a** The relative expression values indicate the relative change in the expression levels between FTAs vs. three normal thyroid glands, assuming that the mean value of the normal samples was equal to 1. U6 was used to normalize RNA levels. The range of variability of the expression of this miRNA in normal thyroid tissues is less than 10%. Each bar represents the mean value ± SE from three independent experiments performed in triplicate. **b** Box plot analysis of miR-130b-3p expression in the same FTAs (t test). * Significance of the difference in miR-130b-3p expression between FTAs and normal thyroids ($p < 0.05$).

pathway, we examined the region encompassing 3,000 bp upstream and downstream of the sequence of miR-130b-3p. We identified a putative CREB recognition site. Subsequently, to evaluate whether the CREB1 protein is able to bind to this miRNA regulatory region in vitro, we performed a ChIP analysis in PC Cl 3 cells treated for 1 h with forskolin (10 μM), and immunoprecipitated chromatin with anti-CREB1 antibodies or rabbit IgG used as control. The results reported in figure 2 show that the CREB1 protein binds to this sequence. In fact, the miRNA region located upstream of the miRNA sequence was amplified from the DNA recovered with anti-CREB1 antibody in PC Cl 3 cells when analyzed by quantitative PCR using specific primers spanning this region. Conversely, no amplification was observed with anti-IgG precipitates (fig. 2). Interestingly, the amount of CREB1 protein bound to this putative regulatory region was much more abundant in cells treated with forskolin than in untreated cells.

miR-130b-3p Is Upregulated in Human Thyroid Adenomas and Contributes to Their Development Targeting CCDC6

We then analyzed miR-130b-3p expression in a panel of FTAs versus normal thyroid tissue. As shown in figure 3, miR-130b-3p was upregulated in 21 out of 23 of FTAs in comparison with normal thyroid tissue.

Since miRNAs are able to modulate gene expression at the posttranscriptional level by directly cleaving mRNAs or repressing mRNA translation [2], we made a search to identify the targets of miR-130b-3p. Using bioinformatic tools (Target Scan, miRWalk and miRanda), we identified several genes that could be targeted by this miRNA. The candidate target of miR-130b-3p was the *CCDC6* gene, which encodes a coiled-coil domain-containing protein and is ubiquitously expressed and may function as a tumor suppressor. Moreover, this protein is responsible for the transcriptional repression of CREB1 target genes [12]. For the seed sequence, there was a matching sequence in the 3′-UTR of the *CCDC6* gene (fig. 4a). To determine the effects of miR-130b-3p on the CCDC6 target, we transfected this miRNA into the PC Cl 3 cells and performed Western blot analysis to look for changes in CCDC6 protein levels. As shown in figure 4b, a clear decrease in CCDC6 protein levels was observed in the cells treated with this miRNA with respect to the scrambled oligonucleotide-treated cells. Similar results have also been obtained with FRTL5 cells transfected with miR-130b-3p (online suppl. fig. 2). A significant reduction in the *CCDC6* mRNA levels has been observed in the cells transfected with miR-130b-3p, in comparison with a scrambled oligonucleotide (fig. 4c). This result indicates miR-130b-3p has an effect on *CCDC6* mRNA degradation.

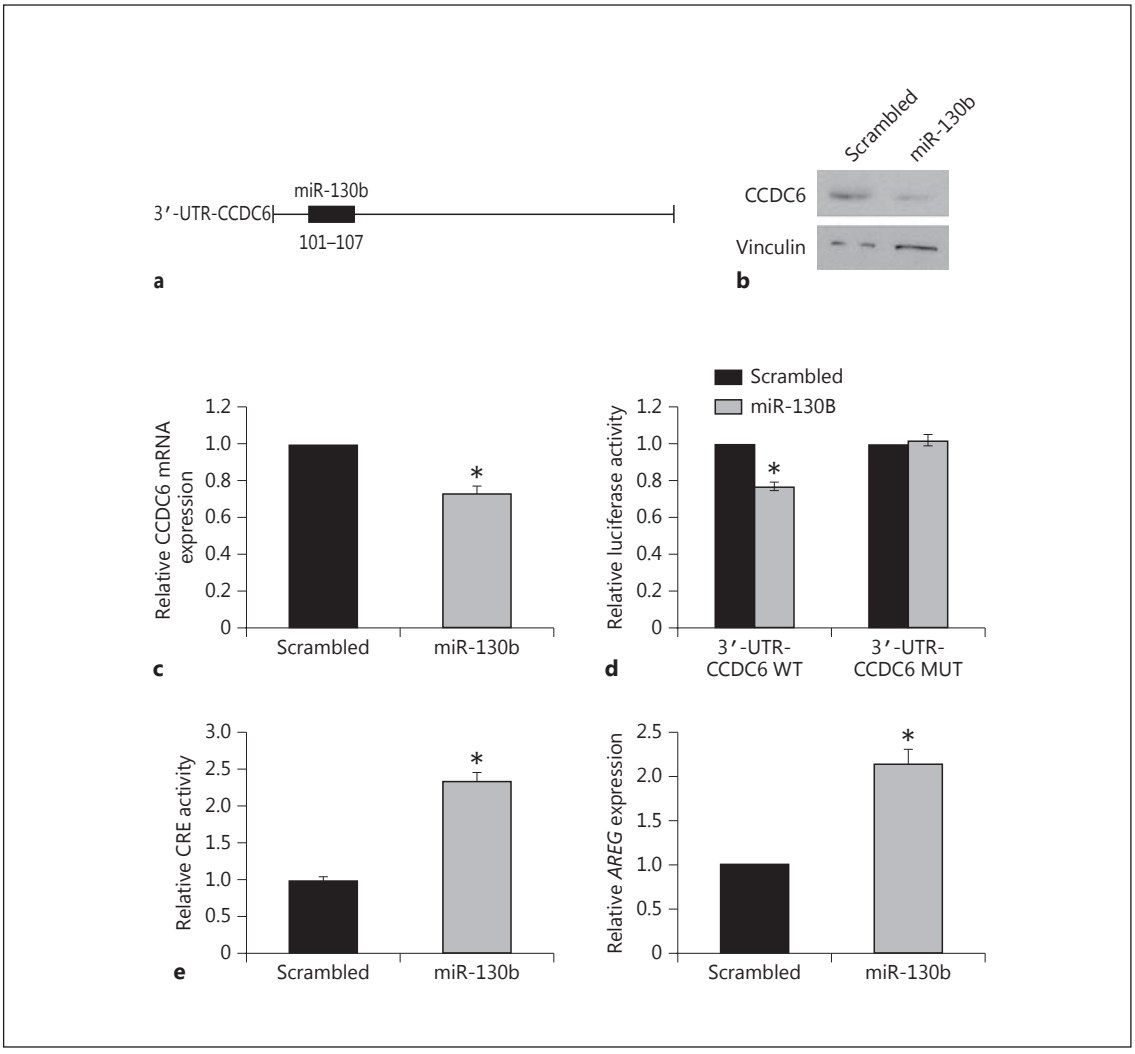

Fig. 4. miR-130b-3p targets CCDC6. **a** Schematic representation of human *CCDC6* 3′-UTR and the relative position of the predicted miRNA-binding site. **b** Western blot analysis of CCDC6 protein levels in PC Cl 3 cells transfected with indicated miRNA precursors or scrambled oligonucleotide and collected at 48 h. Vinculin was analyzed as a loading control. A representative experiment is shown. **c** qRT-PCR analysis of *CCDC6* mRNA in PC Cl 3 cells transfected and collected at 48 h. Relative expression values indicate the relative change in *CCDC6* mRNA expression levels between miR-treated and scrambled oligonucleotide-treated cells, normalized with *G6PD*. The error bars represent the mean ± SE of three independent experiments performed in triplicate. * Significance values of $p < 0.05$ compared to scrambled oligonucleotide-transfected cells. **d** Relative luciferase activity in HEK-293 cells transiently transfected with 3′-UTR-CCDC6 WT and 3′-UTR-CCDC6 MUT along with the indicated miRNA oligonucleotides or with a non-targeting scrambled oligonucleotide. The relative activity of firefly luciferase expression was standardized to a transfection control, using *Renilla* luciferase. The scale bars represent the mean ± SE of three independent experiments performed in triplicate. * Significance values of $p < 0.05$ compared to scrambled oligonucleotide-transfected cells. **e** Relative luciferase activity in PC Cl 3 cells transiently transfected with CRE-Luc reporter and with miR-130b-3p oligonucleotide and a control non-targeting scrambled oligonucleotide (left panel). The relative activity of firefly luciferase expression was standardized to a transfection control, using *Renilla* luciferase. The scale bars represent the mean ± SE (n = 3). * Significance values of $p < 0.05$ vs. the scrambled oligonucleotide. qRT-PCR analysis of *AREG* mRNA in PC Cl 3 cells transfected with miR-130b-3p or the scrambled oligonucleotide (50 nmol/ml) (right panel). Relative expression values indicate the relative change in gene expression levels between miR-treated and scrambled oligonucleotide-treated cells, normalized with *G6PD*. The bars represent the mean ± SE (n = 3). * Significance values of $p < 0.05$ vs. scrambled control.

Eur Thyroid J 2015;4:213–221
DOI: 10.1159/000441355

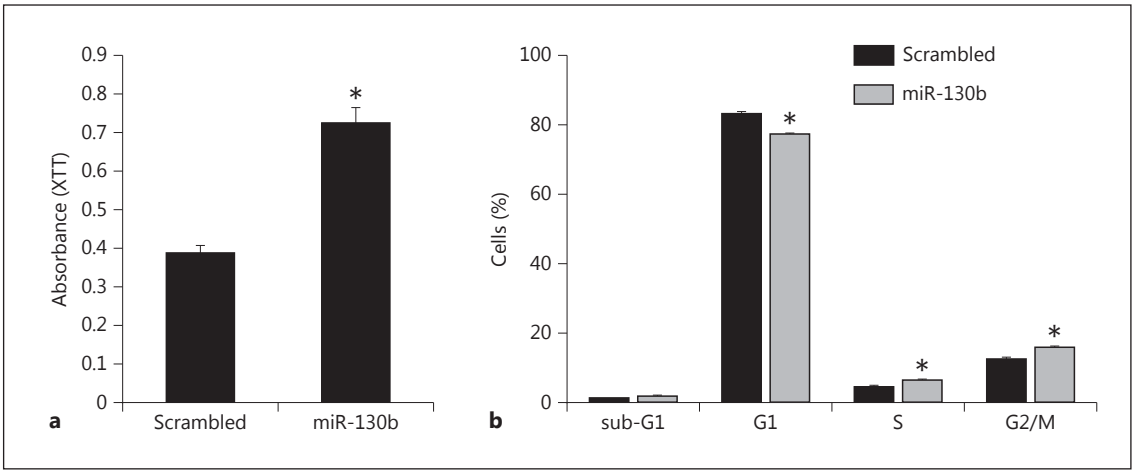

Fig. 5. miR-130b-3p induces cell proliferation. **a** Cell proliferation assay of PC Cl 3 cells transfected with miR-130b-3p and seeded in 96-well plates at 1×10^4 cells/well. After 72 h, 20 μl of Promega's CellTiter® 96 AQueous One Solution were dispensed into each well and absorbance was measured at 595 nm to evaluate cell viability. Each bar represents the mean values ± SE from three independent experiments performed in triplicate. * Significance values of $p < 0.05$ compared to scrambled oligonucleotide-transfected cells. **b** Flow cytometric analysis of synchronized PC Cl 3 cells transfected with miR-130b-3p or scrambled oligonucleotide. After transfection, the DNA of the transfected cells was analyzed 72 h later by flow cytometry after propidium iodide staining. Each bar represents the mean ± SE from three independent experiments performed in triplicate. * Significance values of $p < 0.05$ compared to scrambled oligonucleotide-transfected cells.

Most miRNAs are thought to control gene expression by base pairing with the miRNA-recognizing elements (miR-RE) located in their messenger targets. To determine whether direct interaction between miRNA and *CCDC6* mRNA results in decreased expression of the CCDC6 protein, we inserted 290 bp (37–327) of the 3′-UTR of the *CCDC6* mRNA downstream of the luciferase ORF either in sense (3′-UTR-CCDC6) or in antisense (3′-UTR-CCDC6 MUT) orientation. These reporter vectors were transfected in HEK-293 cells together with miRNA oligonucleotide precursors or a scrambled oligonucleotide. As shown in figure 4d, the luciferase activity of the Luc-sense-3′-UTR constructs was decreased after transfection of miR-130b-3p, compared with the scrambled oligonucleotide, while the luciferase activity of Luc-antisense-3′-UTR constructs did not vary. This indicates that this miRNA interferes with *CCDC6* translation through direct interaction with its 3′-UTR (fig. 4d). Since we have previously demonstrated that CCDC6 leads to the transcriptional repression of CREB1 target genes, we analyzed the activity of a promoter responsive to CREB signaling such as CRE-Luc. As shown in figure 4e left panel, miR-130b-3p overexpression resulted in an increased CREB1-mediated transcriptional activity with respect to the scrambled oligonucleotide-transfected cells. Expression levels of *AREG*, a CREB1 target gene, are significantly higher in PC Cl 3 cells transiently expressing miR-130b-3p with respect to the control cells (fig. 4e, right panel).

miR-130b-3p Overexpression Promotes Proliferation of Rat Thyroid Cells

The next step of our work was to investigate the role of miR-130b-3p in thyroid cell proliferation. First, we evaluated the growth potential of PC Cl 3 cells transiently expressing the miR-130b-3p using an XTT assay 72 h after seeding. As shown in figure 5a, the growth rate of PC Cl 3 cells expressing miR-130b-3p was significantly higher in comparison to the cells transfected with a scrambled oligonucleotide. Then we investigated the cell cycle phase distribution of the miR-130b-3p-transfected cells. As shown in figure 5b, the S and G2-M phases population increased in PC Cl 3 cells expressing miR-130b-3p with respect to the control scrambled oligonucleotide-treated PC Cl 3 cells. Similar results were also obtained with FRTL5 cells (online suppl. fig. 3). Therefore, these results demonstrate that miR-130b-3p is able to exert a positive effect on thyroid cell proliferation.

miR-130b-3p Upregulation Is Associated with a Decrease in CCDC6 Protein Levels in FTAs

In order to further analyze the potential role of miR-130b-3p upregulation in human FTA development, the

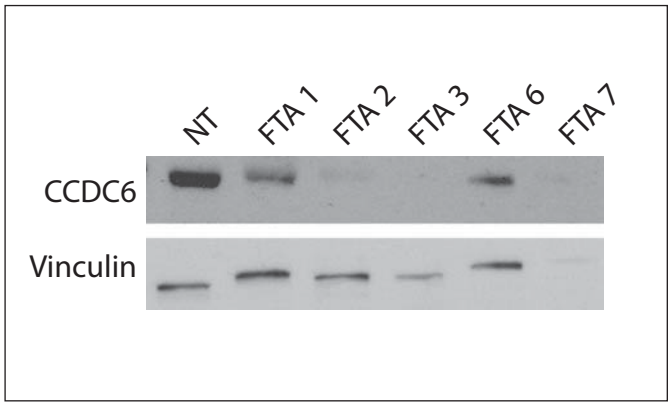

Fig. 6. miR-130b-3p upregulation is associated with a decrease in CCDC6 in human FTAs compared with normal thyroid (NT). Western blot analysis of CCDC6 protein expression in a subset of FTAs. Vinculin was used as loading control.

expression of CCDC6 was studied by Western blot in a subset of FTAs showing a marked upregulation of this miRNA. As shown in figure 6, a decreased CCDC6 protein expression was observed in the analyzed cases, in comparison with normal thyroid tissue. The same result was obtained when CCDC6 mRNA was evaluated in seven FTAs (online suppl. fig. 4).

Discussion

This study aimed to define the role of miR-130b-3p, induced in rat thyroid cells by TSH stimulation, in thyroid cell proliferation and neoplasias. We observed upregulation of miR-130b-3p in most of the FTA samples with respect to normal thyroid tissue. Interestingly, miR-130b-3p was found to be downregulated in papillary, follicular and anaplastic thyroid carcinomas (online suppl. fig. 5). Notably, downregulation of miR-130b expression was recently reported in thyroid carcinomas [18]. The confirmation of these results in a very large number of FTAs and follicular thyroid carcinomas, and hopefully in fine-needle thyroid biopsies from these neoplasias, might open the perspective of the detection of miR-130b-3p as a useful marker for the differential diagnosis between FTA and follicular thyroid carcinoma, which still represents the main challenge in thyroid pathology. On the other hand, this appears to be a unique case where miRNA deregulation shows an opposite behavior depending on the histotype of the tumors deriving from the same cells.

Among the potential candidate genes of miR-130b-3p, we focused on the *CCDC6* gene. Indeed, *CCDC6* has been reported to be fused to *RET* in papillary thyroid carcinomas [19]. Moreover, we recently reported that it interacts with CREB1 and represses its transcriptional activity [12], and Ccdc6-ex2 knock-in mice developed thyroid hyperplasia associated with enhanced CREB1 activity and an increased expression of the CREB1-regulated genes [20], and thus proposed *CCDC6* as a tumor suppressor gene in thyroid neoplasias. Then, we validated CCDC6 as a miR-130b-3p target since the enforced expression of miR-130b-3p significantly decreases CCDC6 RNA and protein levels.

Notably, we demonstrated that the reduced CCDC6 protein levels induced by miR-130b-3p are associated with increased CREB1 activity as indicated by the increase in the expression of the *AREG* gene, a CREB1 target. We then reported that miR-130b-3p directly regulates *CCDC6* mRNA stability and translation since it negatively regulates the expression of a *CCDC6* gene 3′-UTR-based reporter construct and the deletion of the matched site located in the *CCDC6*. Finally, an opposite behavior between CCDC6 protein and miR-130b-3p expression was observed in FTAs, suggesting that the upregulation of this miRNA may contribute to the generation of FTAs. Of course, it cannot be excluded that miR-130b-3p upregulation contributes to the development of FTA by targeting other genes involved in the regulation of thyroid cell proliferation since it is well known that one miRNA is able to target multiple genes.

It is worth noting that miR-130b has been found to be deregulated also in several other human neoplasias. Indeed, miR-130b upregulation has been observed in triple-negative breast cancer, where it mediates the repression of the CCNG2 gene coding for the cyclin G2 protein, a crucial cell cycle regulator [21]. Equally, miR-130b upregulation has been reported in esophageal squamous [22] and colon [23] carcinomas regulating the PTEN-Akt pathway [22]. Interestingly, miR-130b is associated with a poor prognosis in colon [23] and ovarian [24] carcinomas.

Conversely, miR-130b is downregulated in prostate cancer, where its expression is able to decrease cell migration by targeting MMP2, suppressing metastasis [25]. The same tumor suppressor activity is shown by miR-130b in pancreatic cancer by targeting STAT3 [26]. Moreover, we have recently reported that miR-130b was also downregulated in most pituitary adenomas [27].

Therefore, the cell context plays a critical role in determining the oncogenic or tumor suppressor role of miR-130b since several target genes have been identified for miR-130b and likely their modulation accounts for the different effects of miR-130b in cell migration and invasion as it inhibits these functions in prostate [25] and pancreatic [26] carcinomas, whereas these activities are promoted in colon carcinomas [23].

In conclusion, the data reported here support a role of miR-130b-3p upregulation in the development of human FTAs, likely decreasing the CCDC6 protein and, in turn, increasing CREB1 activity.

Acknowledgements

This work was supported by grants from Associazione Italiana per la Ricerca sul Cancro-AIRC (IG 11477), Ministero dell'Università e della Ricerca Scientifica e Tecnologica-MIUR (PRIN 2011), Progetto CREME supported by P.O.R. Campania FSE 2007–2013, CUP B25B09000050007, and the CNR Epigenomics Flagship Project 'EPIGEN'. We are grateful to Mario Berardone for his artwork.

Disclosure Statement

The authors have nothing to disclose.

References

1 Ambros V: The functions of animal microRNAs. Nature 2004;431;350–355.
2 Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004;116:281–297.
3 Chen CZ, Li L, Lodish HF, Bartel DP: MicroRNAs modulate hematopoietic lineage differentiation. Science 2004;303:83–86.
4 Kim VN: MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol 2005;6:376–385.
5 Lewis BP, Burge CB, Bartel DP: Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005;12:15–20.
6 Naguibneva I, Ameyar-Zazoua M, Polesskaya A, Ait-Si-Ali S, Groisman R, Souidi M, Cuvellier S, Harel-Bellan A: The microRNA miR-181 targets the homeobox protein Hox-A11 during mammalian myoblast differentiation. Nat Cell Biol 2006;8:278–284.
7 Hwang HW, Mendell JT: MicroRNAs in cell proliferation, cell death, and tumorigenesis. Br J Cancer 2007;96:R40–R44.
8 Taganov KD, Boldin MP, Baltimore D: MicroRNAs and immunity: tiny players in a big field. Immunity 2007;26:133–137.
9 Stefani G, Slack FJ: Small non-coding RNAs in animal development. Nat Rev Mol Cell Biol 2008;9:219–230.
10 Leone V, D'Angelo D, Ferraro A, Pallante P, Rubio I, Santoro M, Croce CM, Fusco A: A TSH-CREB1-microRNA loop is required for thyroid cell growth. Mol Endocrinol 2011;25:1819–1830.
11 Leone V, D'Angelo D, Pallante P, Croce CM, Fusco A: Thyrotropin regulates thyroid cell proliferation by up-regulating miR-23b and miR-29b that target SMAD3. J Clin Endocrinol Metab 2012;97:3292–3301.
12 Leone V, Mansueto G, Pierantoni GM, Tornincasa M, Merolla F, Cerrato A, Santoro M, Grieco M, Scaloni A, Celetti A: CCDC6 represses CREB1 activity by recruiting histone deacetylase 1 and protein phosphatase 1. Oncogene 2010;29:4341–4351.
13 Ambesi-Impiombato FS, Parks LA, Coon HG: Culture of hormone-dependent functional epithelial cells from rat thyroids. Proc Natl Acad Sci USA 1980;77:3455–3459.
14 Fusco A, Berlingieri MT, Di Fiore PP, Portella G, Grieco M, Vecchio G: One- and two-step transformations of rat thyroid epithelial cells by retroviral oncogenes. Mol Cell Biol 1987;7:3365–3370.
15 De Martino I, Visone R, Wierinckx A, Palmieri D, Ferraro A, Cappabianca P, Chiappetta G, Forzati F, Lombardi G, Colao A, Trouillas J, Fedele M, Fusco A: HMGA proteins up-regulate CCNB2 gene in mouse and human pituitary adenomas. Cancer Res 2009;69:1844–1850.
16 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods 2001;25:402–408.
17 Celetti A, Cerrato A, Merolla F, Vitagliano D, Vecchio G, Grieco M: H4(D10S170), a gene frequently rearranged with RET in papillary thyroid carcinomas: functional characterization. Oncogene 2004;23:109–121.
18 Dettmer MS, Perren A, Moch H, Komminoth P, Nikiforov YE, Nikiforova MN: MicroRNA profile of poorly differentiated thyroid carcinomas: new diagnostic and prognostic insights. J Mol Endocrinol 2014;52:181–189.
19 Grieco M, Santoro M, Berlingieri MT, Melillo RM, Donghi R, Bongarzone I, Pierotti MA, Della Porta G, Fusco A, Vecchio G: PTC is a novel rearranged form of the RET proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell 1990;60:557–563.
20 Leone V, Langella C, Esposito F, Arra C, Palma G, Rea D, Paciello O, Merolla F, De Biase D, Papparella S, Celetti A, Fusco A: Ccdc6 knock-in mice develop thyroid hyperplasia associated to an enhanced CREB1 activity. Oncotarget 2015;6:15628–15638.
21 Chang YY, Kuo WH, Hung JH, Lee CY, Lee YH, Chang YC, Lin WC, Shen CY, Huang CS, Hsieh FJ, Lai LC, Tsai MH, Chang KJ, Chuang EY: Deregulated microRNAs in triple-negative breast cancer revealed by deep sequencing. Mol Cancer 2015;14:36.
22 Yu T, Cao R, Li S, Fu M, Ren L, Chen W, Zhu H, Zhan Q, Shi R: MiR-130b plays an oncogenic role by repressing PTEN expression in esophageal squamous cell carcinoma cells. BMC Cancer 2015;15:29.
23 Colangelo T, Fucci A, Votino C, Sabatino L, Pancione M, Laudanna C, Binaschi M, Bigioni M, Maggi CA, Parente D, Forte N, Colantuoni V: MicroRNA-130b promotes tumor development and is associated with poor prognosis in colorectal cancer. Neoplasia 2013;15:1218–1231.
24 Wang L, Zhu MJ, Ren AM, Wu HF, Han WM, Tan RY, Tu RQ: A ten-microRNA signature identified from a genome-wide microRNA expression profiling in human epithelial ovarian cancer. PLoS One 2014;9:e96472.
25 Chen Q, Zhao X, Zhang H, Yuan H, Zhu M, Sun Q, Lai X, Wang Y, Huang J, Yan J, Yu J: MiR-130b suppresses prostate cancer metastasis through down-regulation of MMP2. Mol Carcinog 2014, Epub ahead of print.
26 Zhao G, Zhang JG, Shi Y, Qin Q, Liu Y, Wang B, Tian K, Deng SC, Li X, Zhu S, Gong Q, Niu Y, Wang CY: MiR-130b is a prognostic marker and inhibits cell proliferation and invasion in pancreatic cancer through targeting STAT3. PLoS One 2013;8:e73803.
27 Leone V, Langella C, D'Angelo D, Mussnich P, Wierinckx A, Terracciano L, Raverot G, Lachuer J, Rotondi S, Jaffrain-Rea ML, Trouillas J, Fusco A: miR-23b and miR-130b expression is downregulated in pituitary adenomas. Mol Cell Endocrinol 2014;390:1–7.