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2-iodohexadecanal Inhibits thyroid cell growth in part through the induction of let-7f microRNA

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ABSTRACT

It is well known that pituitary TSH exerts the major task in the regulation of thyroid function. However, this gland is capable of certain degree of autonomy, independently of TSH control. Iodine plays an important role in thyroid physiology and biochemistry. The thyroid is capable of producing different iodolipids such as 2-iodohexadecanal (2-IHDA). It was shown that this iodolipid mimic some of the inhibitory effects of excess iodide on several thyroid parameters.

Objectives: To identify the miRNAs regulated by 2-IHDA in rat thyroid cells and likely characterize their role in thyroid cell proliferation and function.

Results: FRTL-5 cells were grown in the presence of TSH and treated with 2-IHDA. Among the miRNAs up-regulated by 2-IHDA we focused on miR-let-7f and miR-138. When we transfected the miRNAs, miR-let-7f but not miR-138 overexpression inhibited proliferation of FRTL 5 cells, while miR-let-7f inhibition restored cell growth in 2-IHDA treated cultures. Analysis of cell cycle by flow cytometric DNA analysis revealed that miR-let-7f inhibition reduced the percentage of 2-IHDA treated cells in G1 phase and an increased of the percentage of cells in S phase was observed upon anti-let-7f transfection. The expresion of Cyclin D1 and Cyclin D3 were reduced after the transfection of miR-let-7f and miR-138, respectively. In *in vivo* studies we observed that miR-let-7f and miR-138 were up regulated by 2-IHDA during goiter involution.

Conclusion: These results suggest that the inhibitory effects of 2-IHDA on FRTL-5 thyroid cell proliferation are mediated in part through the induction of let-7f microRNA.

1. Introduction

TSH is the main control of thyroid gland function and growth (Dumont et al., 1992). Other factors, such as iodine play a role in these processes (Panneels et al., 2009; Pisarev and Gärtner, 2000). Since most of these effects of KI are blocked by the addition of methimazol and PTU it was proposed that iodine has to be converted to an intracellular organic (XI) compound in order to exert its inhibitory action (Thyroid cell autoregulation) (Halmi and Stuelke, 1956; Van Sande et al., 1975).

Several candidates have been proposed for XI. The biosynthesis of iodolipids has been observed in the thyroid gland from several species and their participation in thyroid autoregulation has been suggested.

2-iodohexadecanal (2-IHDA) was isolated as the major iodolipid formed in horse thyroid slices incubated *in vitro* with radioiodide (Pereira et al., 1990) while Panneels et al. (1996) have shown the synthesis of this iodocompound in cultured dog thyroid cells. The synthesis of this compound has been demonstrated *in vivo* in rat thyroid after the intraperitoneal injection of KI (Pereira et al., 1990).

We have demonstrated that 2-IHDA has an antigoitrogenic action (Thomasz et al., 2010). Moreover previous results from our lab have demonstrated an inhibitory effect of 2-IHDA on FRTL-5 thyroid cell proliferation mediated by cell Cycle arrest and apoptosis (Thomasz et al., 2015).

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MicroRNA (miRNA or miR) are small noncoding RNA capable of regulating gene expression at translational level. In fact, they repress target gene expression by binding to complementary sequences found in the 3'-untranslated regions (UTR) of target mRNA. Recent evidences suggest that a significant portion of the human genome is regulated by miRNA. One miRNA is capable of regulating several distinct mRNAs, and all the human miRNAs identified so far are believed to modulate more than one-third of the mRNA species encoded in the genome (Ameres and Zamore, 2013; Fabian et al., 2010; Inui et al., 2010). Moreover, each gene may be regulated by more than one miRNA. Therefore, the potential regulatory circuitry afforded by miRNAs is enormous although their role as switchers of gene expression in thyroid cells has been questioned (Floor et al., 2014). The aim of our work has been to identify the miRNAs regulated by 2-IHDA in rat thyroid cells and likely characterize their role in thyroid cell proliferation and function. In the present work, we have carried out miRNA expression profiling of untreated and 2-IHDA treated FRTL 5 cells. Among the miRNA differentially expressed, we focused our attention on miR-let-7f and miR-138 up-regulated in 2-IHDA-treated cells with respect to the untreated cells.

2. Materials and methods

2.1. Cell lines and transfections

FRTL-5 rat thyroid cells (ATTC 8305) were kindly provided by Dr. L.D. Kohn and the Interthyr Research Foundation (MD, USA). Cells were grown in Dulbecco's modified Eagle medium (DMEM/F12, 50:50 v/v) (GIBCO, Invitrogen Corporation, USA) supplemented with 5% fetal bovine serum (FBS) (Natocor, Córdoba, Argentina), bovine thyrotropin (1 mU/mL), hydrocortisone (3.62 ng/ml), transferrin (5 μg/mL), insulin (10 mg/mL), somatostatine (10 ng/ml) and glycil-L-histidyl-L-lysine acetate (10 ng/mL) (6H medium). Cell cultures were kept at 37 °C in 5% CO_2-95% air atmosphere in a humidified incubator. When cultures reached 70% of confluence cells were incubated with 2-IHDA 10 μM for 24 h. The WRO cell line was cultured in RPMI 1640 supplemented with 10% FBS and penicillin (100 U/ml) and were grown in a water-saturated atmosphere containing 5% CO_2 and 95% air at 37 °C.

For transfection of miRNA oligonucleotide, 1×10^6 cells seeded in P60 plates were transfected with 50 nmol/ml premiR miRNA precursor or a control non targeting scrambled oligonucleotide (Ambion, Austin, TX) using siPORT neoFX Transfection Agent (Ambion) according to the manufacturer's instructions. To inhibit miRs expression in the cell lines, anti-miR miRNA Inhibitor (Applied Biosystems) was transfected at 50 nM concentrations using siPORT neoFX Transfection Agent (Ambion).

2.2. RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cell cultures with Trizol (Life Technologies, Inc., Carlsbad, CA) according to the manufacturer's instructions. RNA was reverse transcribed using the Superscript II reverse transcriptase (Invitrogen) or TaqMan MicroRNA Reverse Transcription kits (Applied Biosystems). Real-time PCR was carried out with SYBR Green PCR Master Mix (Biodynamics) or with TaqMan MicroRNA Assays (Applied Biosystems) and TaqMan Universal PCR Master Mix (Applied Biosystems) for miRNA according to manufactures instructions. Expression levels were based on the amount of the target messenger relative to that of the GAPDH transcript or the U6 small nuclear RNA used to normalize RNA levels. To calculate the relative expression levels, we used the $2^{-\Delta\Delta CT}$ method.

2.3. miRNACHIP microarray

Microarray experimental procedures (245 miRNAs) were performed as previously described (Liu et al., 2004). The experiment has been performed three times. Each sample has been analyzed for miRNA expression profile three times.

2.4. Plasmids and constructs

The 3′UTR regions of the Cyc D1, including binding sites for miR-let-7f were amplified by PCR from genomic DNA by using the following primers:

3′UTR-Cyc D1 forward, AATTTCTAGAGGGGGCGTAGCATCATAGTA

3′UTR-Cyc D1 reverse, AATTTCTAGAGTGCAACCAGAAATGCACAG

The 3′UTR regions of the Cyc D3, including binding sites for miR138 were amplified by PCR from genomic DNA by using the following primers:

3′UTR-Cyc D3 forward, AATTTCTAGAACATGGCCAGTCAGTTCCTC

3′UTR-Cyc D3 reverse, AATTTCTAGACTGAAGGACCCAGATCCAAA

The amplified fragments were cloned into pGL3-control firefly luciferase vector (Promega, Madison, WI) at the XbaI site immediately downstream from the stop codon of luciferase in sense orientation. For the generation of 3′UTR constructs mutated in the miR binding sites, the 3′UTR amplified fragments were cloned in antisense orientation.

2.5. Luciferase target assays

FRTL 5 cells were cotransfected using siPORT neoFX transfection agent (Ambion) in 12-well plates with the modified firefly luciferase vector (0.2 μg) described above, the Renilla luciferase reporter plasmid (pRL-CMV, Promega) (30 ng), and the RNA oligonucleotides (50 nmol/ml). Firefly and Renilla luciferase activities were measured 24 h after transfection with the dual-luciferase reporter assay system (Promega). Firefly activity was normalized to Renilla activity as control of transfection efficiency.

2.6. Protein extraction, western blotting, and antibodies

The cells were scraped in ice-cold PBS, and, subsequently, lysed in ice-cold NP40 lysis buffer (0.5% NP40, 50 mM HEPES (pH 7), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, Complete inhibitor (Roche)). Measurement of protein concentration was done using Bradford (Bio-Rad Laboratories, Hercules, CA). Proteins were analyzed on polyacrylamide gel, transferred onto nitrocellulose membranes (Bio-Rad), incubated with specific primary antibodies, and visualized using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA). The antibodies used in this work were: anti-Cyc D1 (sc-8396, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-Cyc D3 (sc-182, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and anti-actina (sc-1615, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

2.7. MTT assay

Transfected cells using siPORT neoFX Transfection Agent (Ambion) were seeded in 96-well plates at 1×10^4 cells/well. After 72 h, 10 μl of MTT (5 mg/ml) was dispensed into each well and absorbance measured at 595 nm to evaluate cell viability. This assay is based on the cleavage of the tetrazolium salt MTT to a dark blue formazan product by mitochondrial dehydrogenase in viable cells.

2.8. Cell counting

Cells transfected with miRs, anti-miRs or the scrambled oligonucleotide (50 nmol/ml) were seeded in P24 plates at 5×10^4 cells per plate. The cells were counted using Neubauer camera 72 h after plating.

2.9. Cell cycle analysis

Cells transfected with miRs or the scrambled oligonucleotide (50 nmol/ml) were seeded in P60 plates at 5×10^5 cells/plate. After 48 h the DNA of the transfected FRTL-5 cells was analyzed. Cell Cycle analysis was performed by propidium iodide (PI) staining. Cells were trypsinized, collected by centrifugation and washed with ice-cold PBS before fixing in 70% ethanol at 4 °C. Fixed cells were resuspended in 0.2 ml PBS containing 50 μg/mL RNase I (Sigma, R4875) and 60 μg/mL PI (Sigma, P4170). Cells were incubated at room temperature for 30 min and then analyzed by flow cytometry on a FACS Calibur (Becton Dickinson Immunocytometry Systems). TSH starved cells were used as a control of G1 arrest. Ten thousand cells were measurement per experimental condition and analyzed with WinMDI and Cylchred software.

2.10. Involution of performed goiter

Goiter was induced by the administration of MMI, 5 mg/day/100 g bw in 0.2 ml of saline for 10 days. At this point MMI was discontinued and the rats were distributed into the following subgroups: a) without further treatment; b) 20 μg/day of 2-IHDA c), 20 μg/day of KI both i.p. The animals were killed 3 days after this last treatment. The ratio thyroid weight/body weight (g) × 100 was determined.

2.11. Materials

All reagents were purchased from Sigma Chemical Co (St. Louis, MO) and 2-IHDA was prepared according to the procedures described by Ohayon et al. (1994).

2.12. Statistical analysis

Statistical analysis was performed using ANOVA followed by Student-Newman-Keuls test for multiple comparisons. Data are expressed as mean ± SEM. Differences were considered significant at $P < 0.05$.

3. Results

3.1. 2-IHDA induces up-regulation of miR-let-7f and miR-138 in FRTL 5 cells

To investigate the role of miRNAs in 2-IHDA effect on thyroid cell growth, RNA extracted from the FRTL 5 cells treated with TSH or TSH+2-IHDA 10 μM for 24 h, were analyzed for the genome-wide miRNA expression profile using a miRNA microarray.

By applying Significance Analysis of Microarray (see Materials and Methods), we obtained a list of miRNAs significantly deregulated in FRTL 5 cells treated with 2-IHDA. In particular, 11 miRNAs were up-regulated and 9 miRNAs were down-regulated with higher than 2-fold change in the FRTL 5 cells treated with 2-IHDA for 24 h in comparison with the same cells treated with TSH alone (Table 1). In this study, we focused on the miR-let-7f and miR-138 which are up-regulated by 2-IHDA and are deregulated in thyroid neoplasias (Pallante et al., 2014). Therefore, we next validated the results of the miRNA microarray analysis by real-time quantitative PCR (qRT-PCR). The qRT-PCR data confirmed the upregulation of let-7f (3.3 fold) and miR-138 (2.7 fold) (Fig. 1). TSH treatment down regulated miR-let-7f while miR-138 was not modified.

Table 1
miRNA differentially expressed between FRTL-5 treated with 2-IHDA 10 μM plus TSH and FRTL-5 treated with TSH.

miRNA	Ratio (treatment TSH + 2-IHDA 10 μM vs TSH)	p-value
hsa-miR-202*	0.006	0.0271750
hsa-miR-556-3p	0.007	0.0197468
hsa-miR-371-3p	0.009	0.0220312
hsa-miR-498	0.010	0.0019256
hsa-miR-543	0.017	0.0247876
hsa-miR-373*	0.017	0.0084601
hsa-miR-93	0.026	0.0143279
hsa-miR-92a	0.037	0.0341023
hsa-miR-663	0.153	0.0391740
hsa-miR-657	6251	0.0346536
hsa-miR-138	7427	0.0318245
hsa-miR-184	8520	0.0108344
hsa-miR-211	10,842	0.0215250
hsa-miR-497*	18,274	0.0422504
hsa-miR-379*	31,204	0.0357114
hsa-miR-601	48,957	0.0324943
hsa-miR-674	60,048	0.0297709
hsa-miR-363	60,701	0.0295473
hsa-miR-519c-5p	67,627	0.0292417
hsa-let-7f	97,023	0.0278886

3.2. miR-let-7f but not miR-138 overexpression inhibits proliferation of FRTL 5 cells

The possible roles of miR-let-7f and miR-138 in thyroid cell growth by the MMT assay were evaluated. To do this, miR-let-7f and miR-138 were overexpressed in FRTL 5 cells by transient transfections. We found that only the FRTL 5 cells, in which miR-let-7f, but not miR-138, has been overexpressed, grew at a slower rate (17%) in comparison with the scrambled oligonucleotide transfected cells (Fig. 2). Cotransfections of miR-let-7f and miR-138 did not alter the results obtained with miR-let-7f alone (results not shown). These results were confirmed counting the cells (Fig. 3a). Analysis of FRTL-5 cells by flow cytometry revealed that the percentage of G1 phase cells increased upon transfection with miR-let-7f, concomitant with a reduction of the cell population in S phase (Fig. 3b). miR-let-7f exposures resulted in a range of approximately

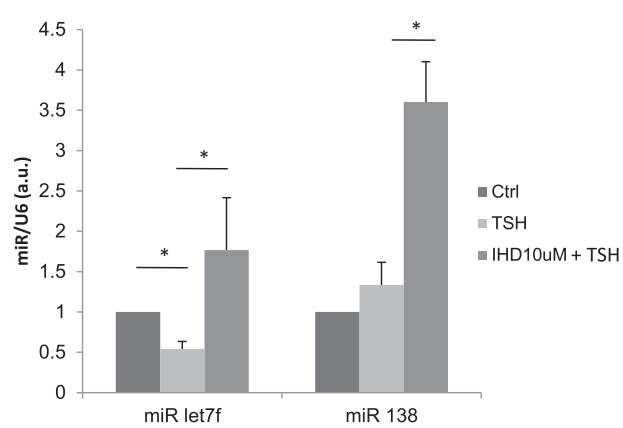

Fig. 1. miR regulation by TSH and 2-IHDA. FRTL-5 cells were treated with 2IHDA 10 μM, in the presence of TSH, for 24 h. The U6 snRNA (U6 spliceosomal RNA) gene was used as an internal control. Data are represented as average of three independent experiments and bars represent ± SEM.

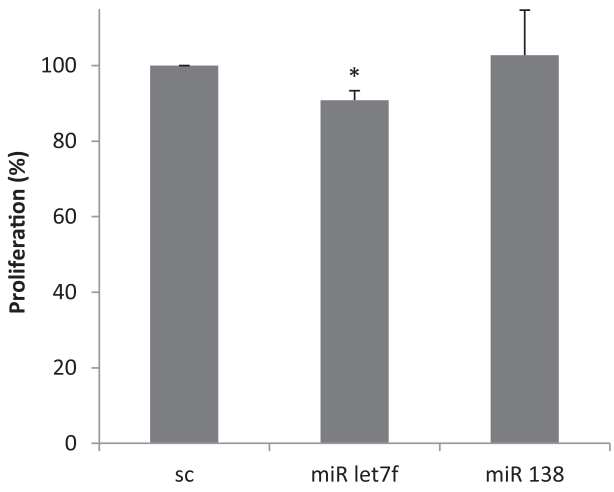

Fig. 2. Cell proliferation assay of FRTL-5 cells transfected with the scrambled oligonucleotide or with miR-let-7f, and miR-138 oligonucleotide (50 nmol/ml) and seeded in 96-well plates at 1×10^4 cells/well. After 72 h, 10 μL of MTT (5 mg/ml) was dispensed into each well and absorbance measured at 595 nm to evaluate cell viability. Each bar represents the mean value SEM from three independent experiments performed in triplicate ($P < 0.05$ compared with the scrambled oligonucleotide).

21% of cells compared with 29% of scrambled treated cells at S phase. No effect was observed with miR-138.

3.3. miR-let-7f inhibition restores cell growth in 2-IHDA treated cultures

To further explore if 2-IHDA-induced miR-let-7f cell growth, FRTL-5 cell cultures were transfected with anti-let-7f or the scrambled oligonucleotide. 24 h later cells were treated or not with 2-IHDA and counted 72 h after plating. We observed that miR-let-7f inhibition suppress 2-IHDA antiproliferative effects and restores cell growth (Fig. 4a). Flow cytometric analysis of FRTL-5 cells showed that miR-let-7f inhibition reduced the percentage of 2-IHDA treated cells in G1 phase and an increased of the percentage of cells in S phase was observed upon anti-let-7f transfection (Fig. 4b).

Taken together these results suggest that 2-IHDA inhibits cell proliferation, at least in part, by inducing let-7f expression in FRTL 5 cells.

3.4. miR-let-7f targets cyclin D1 while miR-138 targets cyclin D3

Since miRNAs are capable to modulate gene expression by targeting mRNA, we used bioinformatic tools (Target Scan, miRDB) to search for mRNA targets of miR-let-7f and miR138. We found several predicted targets involved in many different biological processes, but we focused our attention on Cyclin D1 and Cyclin D3 which have been mentioned as key genes in cell cycle control, contributing to cell proliferation and as we have observed in our lab their expressions are reduced by 2-IHDA.

To validate the influence of miR-let-7f and miR-138 on these targets, we transfected the miR-let-7f or miR-138 oligonucleotides precursors into FRTL 5 cells and we searched for changes in Cyclin D1 or Cyclin D3 mRNA and protein levels by qRT-PCR and western blot analysis, respectively. After the transfection of miR-let-7f, we found an evident reduction in Cyclin D1 mRNA (26%) and protein levels (33%) as compared with the scrambled oligonucleotide (Fig. 5b and c) while miR-138 reduced Cyclin D3 mRNA (30%) and Cyclin D3 protein but at a less extent (14%).

To demonstrate that the direct interaction between the analyzed miRNAs and the Cyclin D1 mRNA was responsible for protein level decrease, we inserted the 3′-UTR of Cyclin D1 and Cyclin D3 mRNAs, including the miR-let-7f and miR-138 seed sites, respectively, downstream the luciferase ORF. The luciferase activities of the Luc-3′UTR constructs were markedly diminished after transfection of miR-let-7f and miR-138, compared with the scrambled oligonucleotide-transfected or untransfected cells, indicating that the reduction of Cyclin D1 protein expression by miR-let-7f and Cyclin D3 by miR-138 was dependent on their direct binding to their 3′-UTR (Fig. 5d).

To study whether miR-let-7f or miR-138 inhibition restores Cyclin D1 and Cyclin D3 expression respectively, we transfected cells with miR-let-7f or miR-138 interfering constructs and then treated them with 2-IHDA. We observed a restoration of Cyclin D1 protein levels or Cyclin D3 upon miR-let-7f or miR-138 inhibition, respectively, when cell were exposed to 2-IHDA (Fig. 5b).

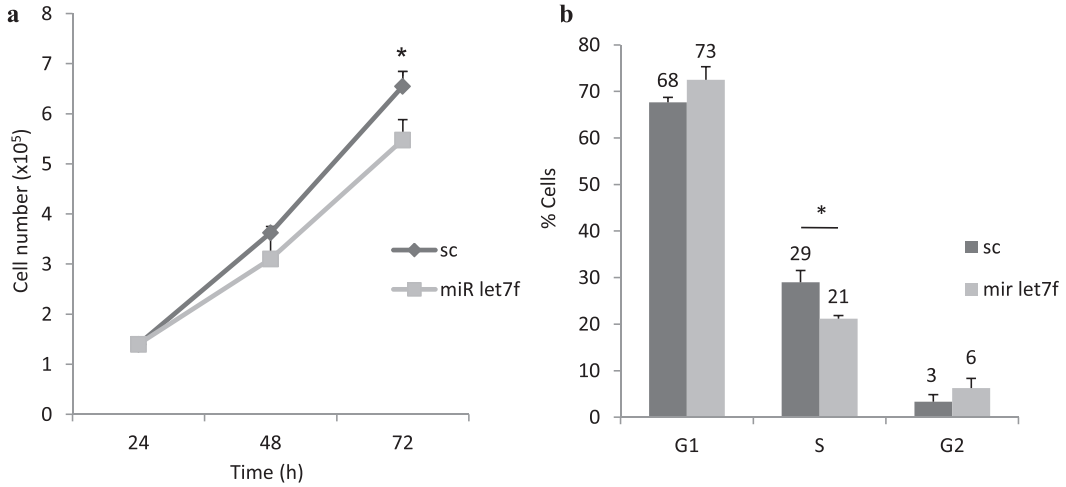

Fig. 3. Overexpression of miR-let-7f inhibits thyroid cell proliferation. **(a)** Cells transfected with miR-let-7f or the scrambled oligonucleotide (50 nmol/ml) were seeded in P24 plates at 5×10^4 cells per plate. The cells were counted 72 h after plating. The y-axis represents absolute viable cell count. Each bar represents the mean values ± sd from three independent experiments performed in triplicate. **(b)** Flow cytometric analysis of FRTL-5 cells transfected with miR-let-7f or the scrambled oligonucleotide. After transfection, the DNA of the transfected FRTL-5 cells was analyzed 48 h later by flow cytometry after propidium iodine staining. Each bar represents the mean ± SEM from three independent experiments performed in triplicate; $P < 0.05$ compared with the scrambled oligonucleotide.

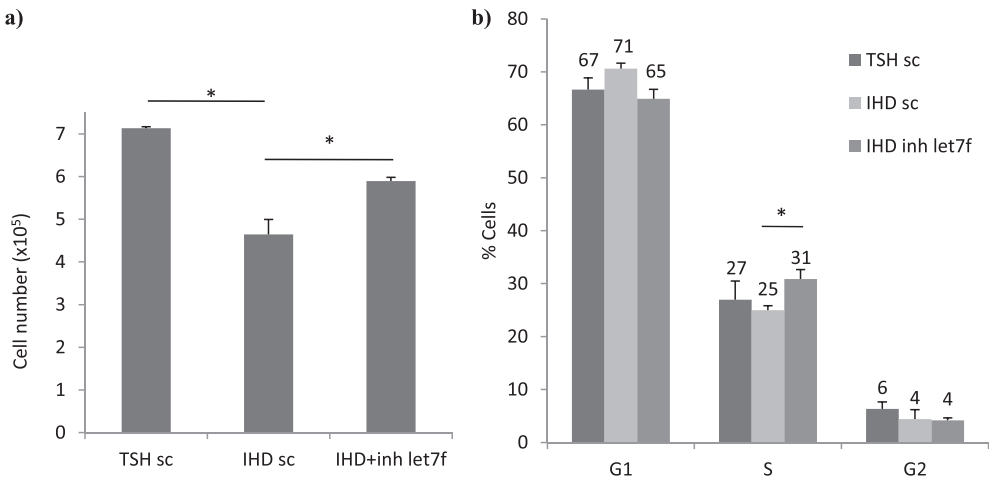

Fig. 4. miR-letf inhibition restores proliferation and cell Cycle progression in FRTL-5 cells treated with 2-IHDA. **(a)** Cells transfected with anti-let-7f (50 μM) or the scrambled oligonucleotide were seeded in P24 plates at 5×10^4 cells per plate. 24 h later were treated or not with 2-IHDA (10 μM). The cells were counted 72 h after plating. The y-axis represents absolute viable cell count. Each bar represents the mean values ± SEM from three independent experiments performed in triplicate. **(b)** Flow cytometric analysis of FRTL-5 cells transfected with anti-let-7f or the scrambled oligonucleotide and 24 h later treated or not with 2-IHDA (10 μM) for 24 h. After each treatment the DNA of the cells was analyzed 24 h later by flow cytometry after propidium iodine staining. Each bar represents the mean ± SEM from three independent experiments performed in triplicate; $P < 0.05$ compared with the scrambled oligonucleotide.

3.5. miR-let-7f and miR-138 regulate specific thyroid gene expression

To further explore the molecular mechanism involved in these miRs effect on thyroid function, mRNA levels for genes involved in thyroid hormone synthesis were measured by qPCR. Fig. 6 shows that the pre-treatment cell line with miR-let-7f, caused a significant reduction (41%) on Tg mRNA levels while the pre-treatment with miR-138 caused a reduction of TPO mRNA (24%). Since transcription factors PAX8, TITF1 and FOXE1 are involved in the regulation of thyroid specific gene expression, their mRNA levels were measured. As can be seen in Fig. 6, miR-let-7f inhibited the expression of FOXE1 (26%) while miR138 inhibited the expression of TITF1 (27%) and FOXE1 (26%). PAX 8 expression was not modified.

3.6. miR-let-7f and miR-138 are up regulated by 2-IHDA during goiter involution

Administration of MMI, 5 mg, during ten days caused a significant increase in thyroid weight by 132%. MMI treatment did not cause a change in miR let-7f or miR-138 levels (Fig. 7a). At this point MMI was discontinued and the rats were distributed into three groups as described in Materials and Methods. The animals were killed after 3 days of these last treatments. Administration of 2-IHDA led to a reduction of 69.7% in goiter while spontaneous involution was only of 39.5% and KI failed to alter this value. miR let-7 f and miR-138 were up regulated 3.0 and 2.9 fold respectively after 72 h of 2-IHDA treatment respect to spontaneous involution. KI did not alter these values (Fig. 7a). Regarding cyclins, after 72 h of involution, 2-IHDA led to a reduction of 51.6% in Cyclin D1 and 40.9% of Cyclin D3 comparing with spontaneous involution while KI reduced 24.3% Cyclin D1, although not statistically significant, and. 27.5% Cyclin D3 mRNA levels (Fig. 7b).

3.7. Thyroid cell autoregulation is lost in dedifferentiated cells

miRs levels were measured in WRO cells (derived from a thyroid follicular cancer). miR-let-7f is slightly decreased (20%) while miR-138 was strongly decreased (96%) comparing with normal thyroid tissue from surgical specimens (Fig. 8a).

2-IHDA failed to inhibit WRO cell growth. It had, although statistically significative, a negligible effect (% $p < 0.01$). No effects of 2-IHDA on miRNAs levels were observed (results not shown).

The roles of miR-let-7f and miR-138 in WRO cell growth were evaluated. We found that only the WRO cells, in which miR-138 but not miR-let-7f have been overexpressed grew at a slower rate (18%) in comparison with the scrambled oligonucleotide transfected cells (Fig. 8b).

4. Discussion

Iodine is not only used by the thyroid to synthesize thyroid hormones but also directly influences a number of parameters such as thyroid proliferation and function. Thyroid autoregulation has been related to intraglandular content of an unknown putative iodocompound. Therefore, an organic iodocompound, called XI, was proposed to be the intermediate of its action (Van Sande et al., 1975). First attempts to identify this putative intermediate have been unsuccessful but iodinated lipids have been found in glands from different species and they reproduce some of the effects of KI. Among them, the thyroid is capable of producing different iodolipids such as 6-iodo-deltalactone (ILδ) and 2-iodohexadecanal (2-IHDA). Data from different laboratories have shown that these iodolipids can inhibit several thyroid parameters suggesting that these compounds may be the intermediates in the thyroid autoregulation process (Panneels et al., 2009; Pisarev and Gärtner, 2000).

Our previous results have shown that 2-IHDA has anti-goitrogenic activity, decreasing the intracellular levels of cAMP, reducing the number of cells and the glandular epithelial height (Thomasz et al., 2010). On the other hand, a direct inhibition of thyroid growth caused by 2-IHDA was observed in *in vitro* cell culture studies with FRTL-5 cells. These inhibitory effects are mediated by cell cycle arrest in G1/S phase and cell death by apoptosis. In the same system we have shown that 2-IHDA negatively regulates the effect of TSH on thyroid-specific gene expression (to be published).

In order to study possible intermediates in the action of 2-IHDA we focused our attention on miRNAs which are small, non-coding endogenous RNAs ~22 nucleotides in length, that regulate gene expression by directing their target mRNAs for degradation or

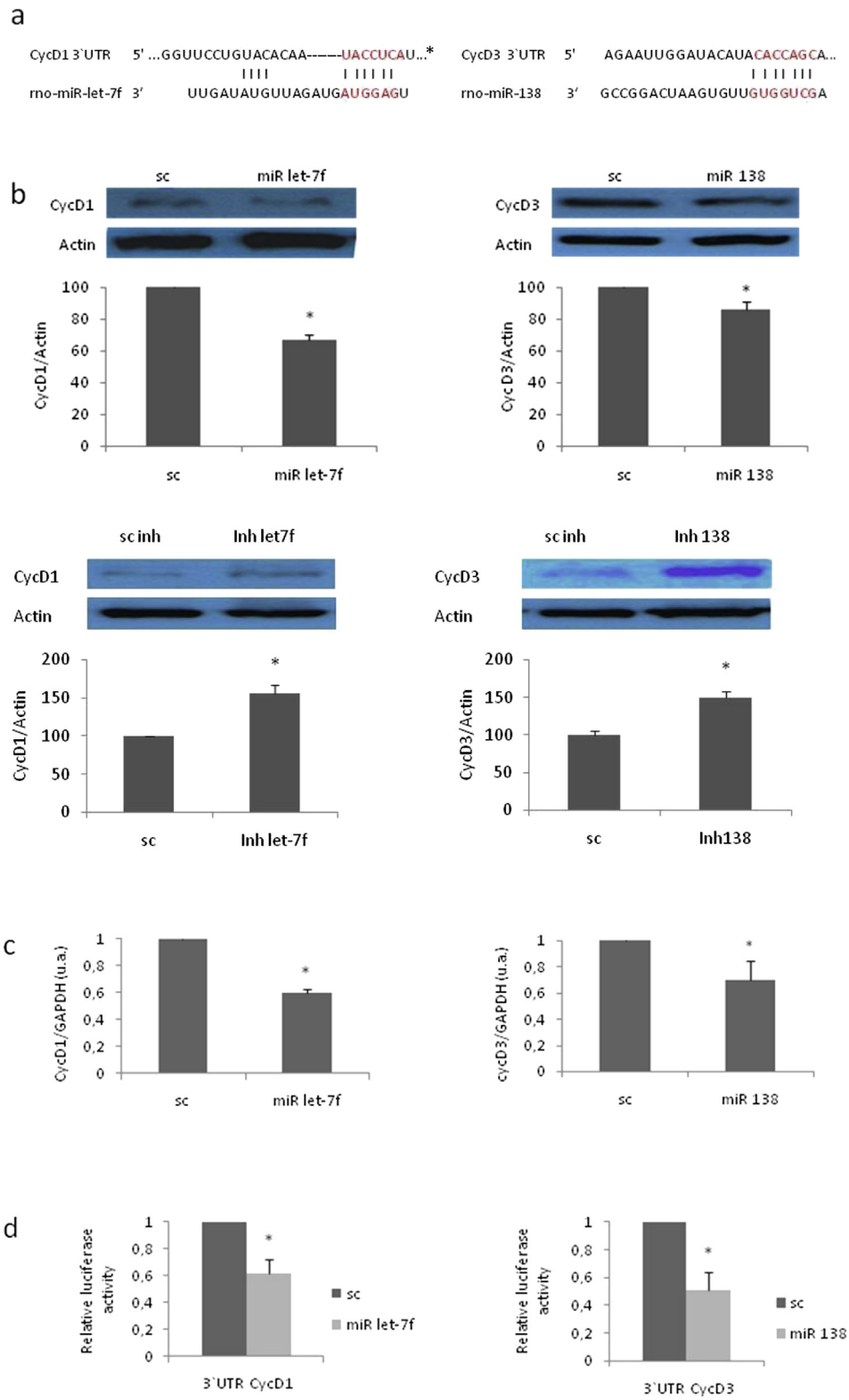

Fig. 5. Post-transcriptional repression of Cyc D1 and Cyc D3 by miR-let-7f and miR-138. **(a)** The predicted binding sites of miR-let-7f on Cyc D1 3′UTR (left panel) and miR-138 on Cyc D3 (right panel) were cloned in pGL3 plasmid, generating the pGL3-Cyc D1-3′UTR-wt plasmid and the Cyc D3-3′UTR-wt plasmid respectively. **(b)** Immunoblots of the Cyc D1, Cyc D3 and actin proteins used as loading control. One of three representative independent experiments is shown. The lower panel represents the densitometric analysis of three independent experiments. Proteins were extracted from FRTL-5 cells transfected with scrambled oligonucleotide, miR-let-7f (upper left panel), miR-138 (upper right panel) or scrambled inhibitor oligonucleotide and the anti-miR-let-7f (bottom left panel) or the anti-miR-138 (bottom right panel) and then treated with 2-IHDA (10 μM) for 24 h and collected after 72 h. **(c)** qRT-PCR analysis of Cyc D1 and Cyc D3 mRNA in the same samples shown in b. Relative expression values indicate the relative change in Cyc D1 and Cyc D3 mRNA expression levels between miR-treated and scrambled oligonucleotide-treated cells, normalized with glyceraldehyde-3-phosphate dehydrogenase. The bars represent the mean ± SEM ($n = 3$) ($P < 0.05$ vs. scrambled control). **(d)** Relative luciferase activity in FRLT-5 cells transiently transfected with wild-type for miRNA seed sequences and with miR let7f oligonucleotide (left panel) or with miR-138 oligonucleotide (right panel) and a control no-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 ± SEM ($n = 3$) ($P < 0.05$ vs. the scrambled oligonucleotide).

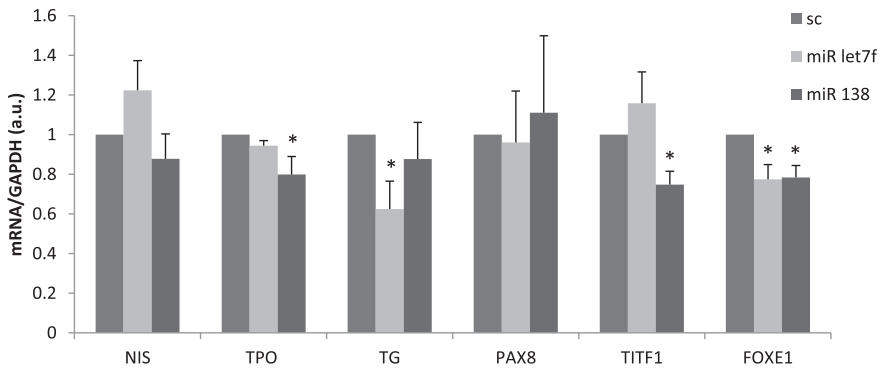

Fig. 6. miR-let-f and miR-138 regulate the expression of thyroid specific genes. FRTL-5 cell lines were transfected with pre-miR-let-7f, miR-138 or the scrambled oligonucleotide and expression of genes was evaluated by qRT-PCR analysis. Results were expressed as the mean ± SEM (n = 3). *P < 0.05; relative to scrambled control.

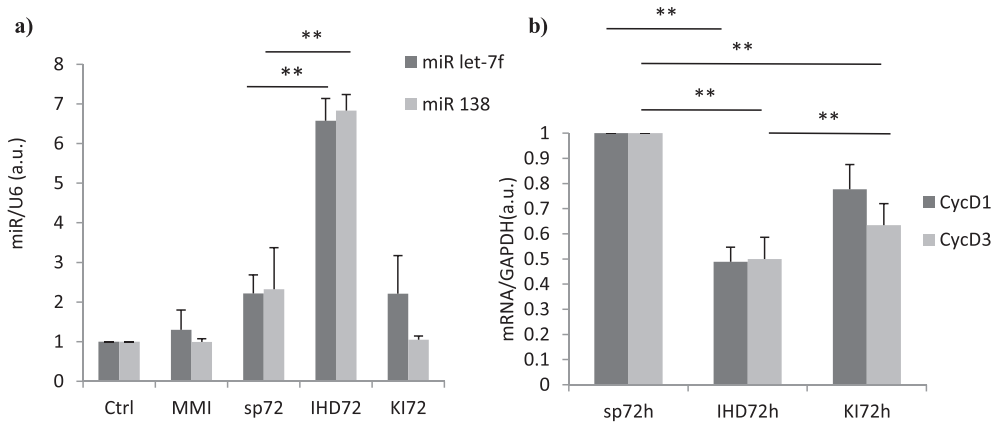

Fig. 7. Effect of 2-IHDA on miRs **(a)** and Cyclins expression **(b)** during goiter formation and involution. The rats were treated with MMI (5 mg/day) or saline solution (CTRL) during 10 days. Then the animals were treated with 2-IHDA (20 μg/d), KI (20 μg/d) or saline solution (spontaneous involution) and sacrificed 3d later. Cyclin expression levels were determined by qRT-PCR. Each value represents the mean of ±SEM of three independent experiments (4 animals each). *P < 0.05; **P < 0.01.

translational repression. Therefore, we have carried out miRs expression profiling of untreated and 2-IHDA treated FRTL 5 cells. The analysis of the miRs expression profile in 2-IHDA treated FRTL-5 cells also showed the up-regulation and down regulation of a set of miRNA. In this study we analyzed the role of miR-let-7f and miR-138. These two miRs are deregulated in thyroid neoplasias (Pallante et al., 2014). Regarding miR-let-7f, one of the most abundantly expressed miRNAs in normal human thyroid gland (Marini et al., 2011), is a regulator of cell cycle, proliferation, and apoptosis, there are conflictive results. Indeed, it was shown an increase in its expression in some tumors (Geraldo et al., 2012; Visone et al., 2007) while in others, the more aggressive, a reduction was reported (Braun et al., 2010; Geraldo et al., 2012; Hébrant et al., 2014; Pallante et al., 2014). miR-138 is down regulated in several types of thyroid neoplasias or thyroid carcinoma cell lines (Braun et al., 2010; Dettmer et al., 2013; Floor et al., 2014; He et al., 2005; Mitomo et al., 2008; Visone et al., 2007; Vriens et al., 2012; Yip et al., 2011); suggesting that these two miRNAs may behave as tumor suppressor genes. Moreover, down regulation of miR-138 is associated with overexpression of human telomerase in human

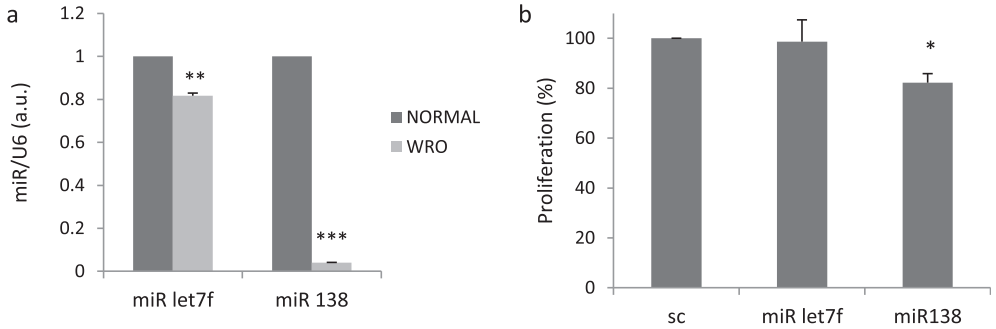

Fig. 8. miR138 restoration inhibits WRO cell growth. (a) Down-regulation of miR-let-7f and miR-138 in WRO cell line as compared with normal thyroid samples. **p < 0.01; ***p < 0.001. (b) Cells transfected with miR-let-7f, miR138 or the scrambled oligonucleotide (50 nmol/ml) were seeded in 96-well plates at 5×10^3 cells/well. After 72 h, 10 μl of MTT (5 mg/ml) were dispensed into each well and absorbance measured at 595 nm to evaluate cell viability. *p < 0.05.

anaplastic thyroid carcinoma cell lines (Mitomo et al., 2008).

miR-let-7f and miR-138 were stimulated by 2-IHDA with an increased fold change with respect to the untreated FRTL-5 cells. When we studied the growth potential of FRTL-5 cells transiently expressing miR-let-7f or miR-138 we observed that only the overexpression of miR-let-7f inhibited cell proliferation and this inhibition was due to an arrest of the cell in the G1/S phase of the cell cycle. Moreover miR-let-7f inhibition restored partially cell growth in 2-IHDA treated cultures. Although a significant effect over cell proliferation and protein expression was observed upon miRNAs transfection these effects were weak. Two main reasons could explain this. Iodolipids effect implies the concerted expression of several miRNAs, a synergic effect that our methodology, i.e. functional analysis of selected miRNAs, was not able to show. However a partial effect as we have shown implies that at least these miRNAs mediate partially iodolipid effects. On the other hand this could be explained also by some characteristics of miRNAs regulation, i.e. the multiplicity of mRNA targets for each miRNA and the multiplicity of miRNA regulating each mRNA, supporting likewise their role as long-term fine tuners, "attenuators" rather than switches of gene expression (Floor et al., 2014).

As miRNAs usually induce gene silencing by binding to target sites found within the 3′UTR of the targeted mRNA, using bioinformatics tools, we identified Cyclins D1 and D3 as candidates targets of miR-let-7f and miR-138 respectively. Cyclins and CDKs play important roles in thyroid cell Cycle regulation (Roger et al., 2010). Cyclin D1 and Cyclin D3, member of the D-type family of G1 Cyclins regulate G1 progression in mammalian cells and TSH induces their expression or activates their complex with CDK4 (Motti et al., 2003; Paternot et al., 2006; Yamamoto et al., 1996). Cyclin D3 is more specifically involved in TSHr/cAMP pathway in thyrocytes (Depoortere et al., 1998; Motti et al., 2003; Paternot et al., 2006) and cAMP-dependent stimulation of the activity of Cyclin D3-CDK complexes is involved in the pathogenesis of human thyroid goiter and adenoma (Roger et al., 2010). We have previously shown that the expression of Cyclin A, Cyclin D1 and Cyclin D3 were reduced after treatment with 2-IHDA. We, therefore, determined the effect of miR-let-7f and miR-138 on protein expression of Cyclins D1 and D3, respectively. The transfection of oligonucleotide precursors of miR-let-7f miRs and 138 lead to a negative regulation of the expression of Cyclins D1 and D3 respectively. It is likely that these miRs reduce Cyclins levels by affecting theirs mRNA stability. Unexpectedly miR-138 didn't inhibit thyroid cell proliferation despite an effect of the miR on Cyclin D3 levels. Perhaps the percentage of inhibition of the cyclin was not sufficient to observe an effect on proliferation. Other difference is that miR-let-7f is down regulated by TSH after 24 h of treatment, agreeing with Akama et al. (2012) but not miR-138. Floor et al. showed that mir-138 is down regulated after 72 h of TSH treatment in primary thyrocytes cultures obtained from autonomous adenomas (Floor et al., 2014). The discrepancy with our results may be attributed to the different models employed (time and cells).

In *in vivo* studies we have shown that 2-IHDA was able not only to prevent the growth of MMI induced goiter but also caused the involution of performed goiter. The iodolipid caused a significant reduction in goiter weight after 3 days compared with the spontaneous involution and iodide failed to alter this value. After treatment of MMI, ten days, no modification of miRs was observed; 3 days later after involution an increase of miR-let-7f and miR-138 levels was only observed in the thyroids from rats injected with 2-IHDA. Riesco et al., have shown that a balanced decrease in cellular proliferation and increase of apoptotic phenomena is involved in the goiter involution (Riesco et al., 1998). miR-let-7f is well known miR which participate in diverse biological functions including development, cell proliferation, differentiation, and apoptosis (Boyerinas et al., 2010). It was also demonstrated a role of miR-138 in apoptosis (Chakrabarti et al., 2013; Gong et al., 2013; Xu et al., 2014), suggesting a role of these miRs in goiter involution.

miR-let-7f and miR-138 also regulated thyroid specific genes. Tg expression was diminished by miR-let-7f while TPO and the transcriptions factors TITF1 and FOXE1 were down regulated by miR-138. These transcription factors are not only indispensable for the correct developing of the thyroid gland but also for the maintenance of the differentiated thyroid (Frenández et al., 2015). Ricarte-Filho et al. (2009) showed that the stable transfection of TPC1 cells (a papillary thyroid carcinoma cell line) with miR-let-7f showed a reduced expression of Cyclin D1 and an increase in TITF1 which is involved in thyroid organogenesis. A role of miRNAs as acute main switches of gene expression in thyroid cells has been questioned (Floor et al., 2014), but a role of miRNAs, especially at long times, cannot be neglected since thyrocite-specific Dicer (an enzyme responsible for generating miRNAs) knockout alters thyroid follicular organization and prevents goiter development. Moreover these animals which developed mild hypothyroidism, Nkx2-1, thyroglobulin, Paired box 8, and TSH receptor proteins were down-regulated compared with controls (Undeutsch et al., 2015). As miR138 has no effect on cell proliferation, it seems that it is more related to differentiation than proliferation.

Thyroid autoregulation may be defined as the capability of the intracellular content of iodide to modulate the gland function. It is exerted through an iodide organified compound and iodolipids would be such compounds. WRO is a thyroid cell line derived from a follicular thyroid cancer which resembles to *in vivo* undifferentiated tumors (van Staveren et al., 2007). Not only it has a different behavior from normal cells but it is refractory to the action of the iodolipid indicating that thyroid cell autoregulation is lost. In our study we have seen a down regulation of miRs let-7f and 138 in these cells as compared with normal thyroid tissue. Indeed 2-IHDA was not able to produce changes in their expression levels. Nevertheless when we restored miR138 levels an inhibition effect over WRO cell proliferation was observed. Moreover it was showed that overexpression of miR let-7f inhibited proliferation of TPC cells (Ricarte-Filho et al., 2009). We have seen a low effect of 2-IHDA over these cells (unpublished results). Taken together these results suggest that 2-IHDA desensitization of these thyroid carcinoma cells (WRO and TPC) could be explained in part by the lost of miRs regulation by 2-IHDA. Further studies are needed to know where the switch occurs at the molecular level.

In conclusion miR-let-7f partially mediates the effects of 2-IHDA on cell proliferation by repressing the expression of the post-transcriptional level of Cyclin D1. The role of miR-138 is less clear but given that 2-IHDA regulated in concert a wide variety of mirs, the absence of a direct effect of miR-138 on proliferation does not rule out his participation as intermediary of 2-IHDA thyroid cell autoregulation.

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