

Brief Article

Effects of let-7 microRNA on Cell Growth and Differentiation of Papillary Thyroid Cancer¹

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Abstract

Papillary thyroid carcinoma (PTC) is the most common endocrine malignancy and *RET/PTC* rearrangements represent key genetic events frequently associated to this cancer, enhancing proliferation and dedifferentiation by activation of the *RET/PTC*-*RAS*-*BRAF*-mitogen-activated protein kinase (MAPK) pathway. Recently, let-7 microRNA was found to reduce *RAS* levels in lung cancer, acting as a tumor suppressor gene. Here, we report that *RET/PTC3* oncogenic activation in PCCL3 rat thyroid cells markedly reduces let-7f expression. Moreover, stable transfection of let-7 microRNA in TPC-1 cells, which harbor *RET/PTC1* rearrangement, inhibits MAPK activation. As a result, let-7f was capable of reducing TPC-1 cell growth, and this might be explained, at least in part, by decreased messenger RNA (mRNA) expression of cell cycle stimulators such as *MYC* and *CCND1* (cyclin D1) and increased *P21* cell cycle inhibitor mRNA. In addition, let-7 enhanced transcriptional expression of molecular markers of thyroid differentiation such as *TTF1* and *TG*. Thus, reduced expression of let-7f might be an essential molecular event in *RET/PTC* malignant transformation. Moreover, let-7f effects on thyroid growth and differentiation might attenuate neoplastic process of *RET/PTC* papillary thyroid oncogenesis through impairment of MAPK signaling pathway activation. This is the first functional demonstration of an association of let-7 with thyroid cancer cell growth and differentiation.

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Introduction

MicroRNA (miRNA) are noncoding ~22-nucleotide RNA that negatively regulate gene expression at the posttranscriptional level either by directly cleaving targeted messenger RNA (mRNA) or by repressing translation [1–3]. Originally discovered in *Caenorhabditis elegans*, these small RNA are now recognized as one of the major regulatory gene families in plants and animals [4]. Lethal-7 (let-7) is one of the most investigated miRNA, being required for timing of stem cell division and differentiation in *C. elegans* [5,6]. In the human genome, almost 700 miRNA genes have been discovered, and the estimated number of miRNA is as high as 1000 [7,8]. Although the biology of most miRNA is not well understood, countless studies have linked anomalous expression of these molecules to carcinogenesis [1]. In humans, let-7 was shown to contain multiple putative complementary sites in the 3' untranslated region of all three *RAS* genes (*HRAS*, *KRAS*, and *NRAS*), and it functions to negatively regulate their protein levels [9]. Because *RAS* activation is associated with several cancers, let-7 down-regulation or deletion could therefore play a role in tumorigenesis. In fact, let-7 overexpression was found to inhibit lung and colon cancer cell growth *in vitro* [9–11]. These findings underscore the importance

of this small RNA as a tumor suppressor for cancers in which the *RAS* pathway is constitutively activated.

Papillary thyroid carcinoma (PTC) is the most prevalent endocrine malignancy in humans [12]. Along with others, this laboratory has shown that ~70% of PTCs harbor genetic alterations in *RET*, *RAS*, or *BRAF* with practically no overlap, providing genetic evidence that constitutive linear signaling along the *RET*-*RAS*-*BRAF*-*ERK* pathway is key to their development [13,14]. In particular, *RET* gene rearrangements occur in up to 43% of PTCs [15]. These rearrangements

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result in the recombination of the intracellular kinase-encoding domain of *RET* with different activating genes, thereby generating *RET/PTC* fusion oncogenes. Biologic effects mediated by *RET/PTC* include enhanced proliferation and dedifferentiation, and they depend on the activation of the *RET/PTC*-RAS-BRAF-ERK axis [16]. This requirement for mitogen-activated protein kinase (MAPK) activation is supported by experimental evidence demonstrating that depletion of MAPK cascade components, such as RAS or BRAF, interferes with *RET/PTC*-induced ERK phosphorylation [17,18].

In this study, the potential involvement of let-7 miRNA in PTC development is investigated. Because let-7 was demonstrated to inhibit RAS expression, it might provide a tumor suppressor function in thyroid cancers with MAPK activation. Indeed, we show that let-7f induction in TPC-1 cells, a human PTC cell line that spontaneously harbors the *RET/PTC1* oncogene, causes a marked reduction in cell proliferation and induces expression of molecular markers characteristic of thyroid differentiation. Our data suggest that let-7 miRNA is an essential regulator of thyroid carcinogenesis.

Materials and Methods

Cell Culture

PTC3-5 [19] and PCCL-BRAF cells were derived from PCCL3 rat thyroid cells to obtain doxycycline-inducible expression of *RET/PTC3* and *BRAF^{V600E}* oncogenes, respectively. These cells were maintained in Ham's F12 medium supplemented with 5% fetal bovine serum, 1 mIU/ml bovine thyroid-stimulating hormone (Sigma, St Louis, MO), 10 µg/ml insulin (Sigma), 5 µg/ml apotransferrin (Sigma), 10 nM hydrocortisone (Sigma), 100 U/ml penicillin/100 µg/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA), 1 µg/ml amphotericin (Invitrogen Life Technologies), and antibiotics at 37°C in a humidified 5% carbon dioxide incubator. *RET/PTC3* and *BRAF^{V600E}* expression was induced after treating with 1 µg/ml doxycycline (Calbiochem, San Diego, CA) for 72 hours. Cells cultivated in the absence of doxycycline were used as controls.

TPC-1, a human PTC cell line spontaneously harboring the *RET/PTC1* rearrangement [20], was maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, 100 U/ml penicillin/100 µg/ml streptomycin, and 1 µg/ml amphotericin at 37°C in a humidified 5% carbon dioxide incubator.

This study complied with the guidelines from the ethical committee of the Institute of Biomedical Sciences (no. 20/F43/L2), University of São Paulo.

Transfection

TPC-1 cells were cultured to 80% to 90% confluence and transfected with pH1-RNApuro-control or pH1-RNApuro-let-7f plasmids using Lipofectamine 2000 (Invitrogen Life Technologies). Stable cell lines were obtained by culturing the cells with 1 µg/ml puromycin (Calbiochem) for 2 to 3 weeks. Stable clones overexpressing let-7f were screened by quantitative polymerase chain reaction (PCR) and used for further experiments. pH1-RNApuro-control and pH1-RNApuro-let-7f plasmids were kindly donated by Dr Takashi Takahashi (Nagoya University Graduate School of Medicine, Nagoya, Japan).

MTT Assay

Cells were seeded into 96-well plates at a density of 8×10^3 cells per well. When the cells reached semiconfluence, 3-(4,5-dimethylthiazol-2-

yl)-2,5-diphenyltetrazolium bromide (MTT; Molecular Probes, Eugene, OR) was added to the medium at a concentration of 0.125 mg/ml. After 3 hours, the medium was removed, and the cells were solubilized in 100 µl of 0.04 M HCl in isopropanol and the reaction product was measured by a spectrophotometer at 595 nm.

Western Blot Analysis

Cells were washed twice with ice-cold PBS and scraped in RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 0.1% SDS) containing 10% protease inhibitor cocktail (Sigma). Measurement of protein concentration was done using Bradford (Bio-Rad Laboratories, Hercules, CA), and 50 µg of each sample was fractionated by 10% SDS-PAGE and blotted onto a nitrocellulose membrane (Hybond-ECL; Amersham Biosciences, Little Chalfont, UK). Nonspecific binding sites were blocked by incubating with 5% nonfat dry milk in Tris-buffered saline-0.1% Tween-20. The following primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used: anti-ERK1 K23, anti-phospho-ERK E4, anti-H-RAS C20, anti-K-RAS F234, and anti-α-tubulin B7. The antigen-antibody complexes were visualized using horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence system (Amersham Biosciences). Expression was quantified using image densitometry with Scion Image Analysis Software (Scion Corporation, Frederick, MA).

Quantitative PCR

Total RNA was isolated using the phenol-chloroform method with the TRIzol reagent (Invitrogen Life Technologies). Complementary DNA (cDNA) was generated from 3 µg of total RNA in the presence of random hexamers and MMLV reverse transcription (RT; Invitrogen Life Technologies) as described previously [21]. Primers were designed using the program Primer Express (Perkin-Elmer Applied Biosystems, Foster City, CA) as follows: *CCND1 (cyclin D1)*: 5'-CTGTGCATCTACACCGACAACCTC and 5'-CCAGGTTCCACTTGAGCTTGTT; *CDKN1A (P21)*: 5'-CTGGAGACTCTCAGGGTTCGAA and 5'-GGCGTTTGGAGTGGTAGAAATCT; *MYC*: 5'-TTCGGGTAGTGGAAAACCAG and 5'-TCCTGTTGTGAAGCTAACG; *NKX2-1 (TTF1)*: 5'-AGCCTGTCCACCTGAACCT and 5'-ATAGCAAGGTGGAGCAGGACAT; *TG*: 5'-CCTGCTGGCTCCACCTTGTTT and 5'-CCTTGTTCTGAGCCTCCCATCGTT; *RPL19*: 5'-TCTCATGGAACACATCCACAA and 5'-TGGTCAGCCAGGAGCTTCTT. Each set of sense and antisense primers was chosen within distinct exons to avoid amplification of genomic DNA. PCR amplification was performed in triplicate using 50 ng of cDNA, 200 or 300 nM of each primer and SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) in a GeneAmp 7300 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA). PCRs without cDNA resulted in no amplification of any target gene. The expression of each gene of interest was normalized with housekeeping gene *RPL19*. Data were analyzed using the Q-Gene program [22].

let-7 Mature miRNA Quantification

Quantitative analysis of miRNA was accomplished using a real-time PCR assay previously reported [23]. In the first step, a tailed miRNA-specific primer was used to convert the RNA template into the cDNA at 50°C for 30 minutes and at 85°C for 5 minutes (5'-CATGATCAGCTGGGCCAAGAACTATA). Then, the full-length cDNA was quantified by real-time PCR using a combination of a locked

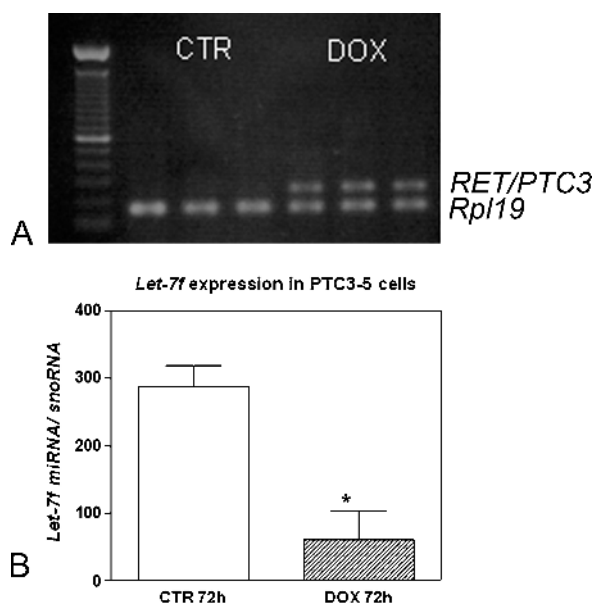


Figure 1. Effect of the constitutive activation of the *RET/PTC3* oncogene on let-7 miRNA expression. (A) Representative gel of the RT-PCR results showing *RET/PTC3* mRNA induction after 72 hours of doxycycline (DOX) treatment. The reactions are normalized by comparison to the *Rpl19* housekeeping gene. γ -Axis represents 100-bp ladder. (B) *RET/PTC3* induction leads to reduced let-7 miRNA expression in PTC3-5 cells. γ -Axis represents *let-7f* expression normalized by *snoRNA*. Columns in the graph represent mean \pm SD of two independent reactions performed in triplicate. * $P = .002$ versus TPC-1 CTR.

nucleic acid (LNA)-containing miRNA primer (5'-T+GA+GGTAG-GTAGATTG, LNA substitutions are preceded by a "+"), and a generic universal primer that is complementary to the tail (5'-CATGATCAG-CTGGGCCAAGA). The LNA modification stabilizes the conformation of the sugar group and thereby increases the hybridization affinity of oligonucleotides that contain LNA bases [24]. Amplification of this chimeric cDNA was monitored by SYBR green real-time PCR under the following conditions: 50°C for 2 minutes, 95°C for 15 seconds and 40 cycles at 95°C for 15 seconds, 54°C for 30 seconds, and 72° for 30 seconds. Data were normalized with the expression of RNU6B (small nuclear RNA) for human cells or small nucleolar RNA (snoRNA) for rat cells using a specific amplification kit (Applied Biosystems, Warrington, UK).

Statistical Analysis

The results are presented as the mean \pm SD and submitted to analysis of variance using the Bonferroni *t*-test. Differences were considered significant at $P < .05$.

Results

Doxycycline-Inducible Expression of *RET/PTC3* in PCCL3 Rat Thyroid Cells

To determine whether the expression of mature let-7f miRNA is modulated by RET/PTC, PTC3-5 cells, derived from PCCL3 rat thyroid cells modified to contain doxycycline-inducible expression of *RET/PTC3*, were analyzed. RT-PCR was used to confirm the efficient induction of *RET/PTC3* expression by treatment of the PTC3-5 cells with doxycycline for 72 hours (Figure 1A). The induced expression of

the *RET/PTC3* oncogene in PTC3-5 cells led to a strong reduction (76%) in let-7f miRNA expression (Figure 1B). However, induction of the *BRAF* oncogene in PCCL3 cells treated with doxycycline for 72 hours (Figure 2A) did not modulate let-7f mature miRNA expression (Figure 2B).

Effects of let-7f Introduction on MAPK Signaling Pathway in TPC-1 Cells

The observation that let-7f expression decreases in response to RET/PTC activation led us to explore the possibility that let-7f-reduced expression might contribute to papillary thyroid cancer development. To test this idea, let-7f was introduced into the TPC-1 cells, which spontaneously harbor the *RET/PTC1* rearrangement, using expression constructs designed to synthesize mature miRNA of the let-7f-1 isomer under the control of the RNA polymerase-III H1-RNA gene promoter [11]. Real-time PCR was used to evaluate the expression of let-7f mature miRNA in TPC-1 control (CTR) and TPC-1 let-7 stable clones. let-7f was observed to have a basal expression level in TPC-1 cells, which was substantially increased after introducing the let-7f plasmid (Figure 3A). Because RET/PTC activation sequentially triggers stimulation of RAS, BRAF, and ERK to mediate its biologic effects, the potential influence of let-7f overexpression on ERK activation was evaluated. As expected, results of the Western blot analysis demonstrated that let-7f induction resulted in a decrease in phosphorylation of the ERK protein in TPC-1 cells (Figure 3B),

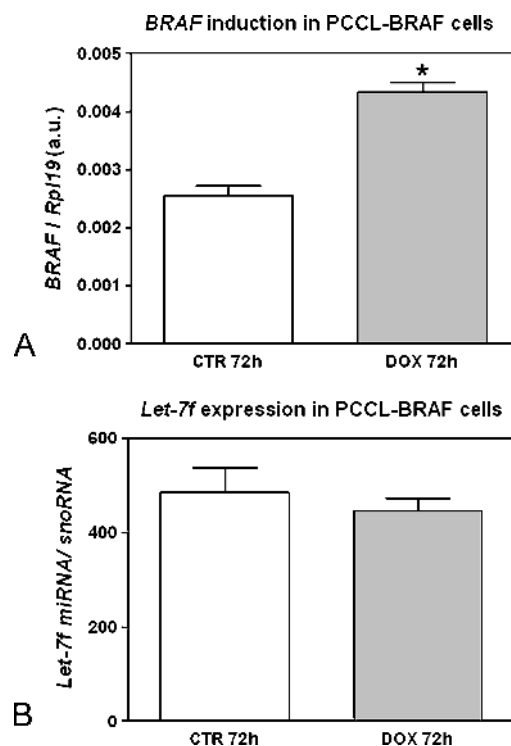


Figure 2. Effect of constitutive activation of the *BRAF* oncogene on let-7 miRNA expression. (A) *BRAF* induction in PCCL-BRAF cells after 72 hours of DOX treatment. γ -Axis represents *BRAF* expression normalized by *Rpl19*. (B) let-7 expression does not fluctuate even when *BRAF* is induced for 72 hours. γ -Axis represents *let-7f* expression normalized by *snoRNA*. Columns in the graph represent result as mean \pm SD in arbitrary units (a.u.) of two independent quantitative RT-PCRs performed in triplicate. * $P = .001$ versus TPC-1 CTR.

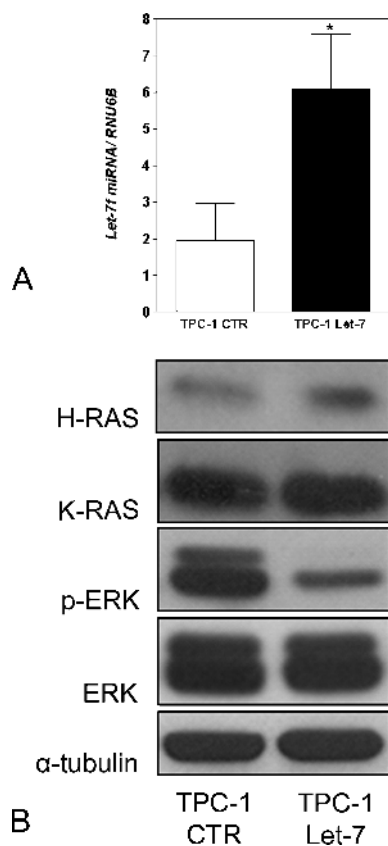


Figure 3. Stable transfection of let-7 miRNA in TPC-1 cells. (A) Quantitative RT-PCR detection of the expression of let-7 miRNA in cells stably transfected with pH1-RNApuro-CTR and pH1-RNApuro-let-7f-1. γ -Axis represents *let-7f* expression normalized by *RNU6B*. Columns in the graph represent mean \pm SD of two independent reactions performed in triplicate. (B) Total cellular proteins were prepared and subjected to 10% SDS-PAGE electrophoresis. Immunoblot analyses were performed using specific antibodies against K-RAS, H-RAS, phosphorylated ERK (p-ERK), ERK1/2, and α -tubulin. * $P = .017$ versus TPC-1 CTR.

even without altering H-RAS and K-RAS protein levels (expression quantified by densitometry and normalized by tubulin levels).

Growth Inhibitory Effect of let-7f Mature miRNA on TPC-1 Cells

The MTT assay was used to examine whether let-7f expression is associated with PTC cell growth. let-7f overexpression markedly inhibited proliferation of TPC-1 cells by 55% (Figure 4). To identify genes involved in the inhibition of TPC-1 cell growth, the transcriptional expression of genes known for their action in the cell cycle, such as *MYC*, *CCND1* (*cyclin D1*), and *CDKN1A* (*p21*), was examined. let-7f inhibited the mRNA expression of cell cycle stimulators such as *MYC* (17%) and *CCND1* (14%). However, this small RNA also reduced the transcriptional expression of *CDKN1A* (18%), a gene that acts as a cell cycle inhibitor (Figure 5).

Effects of let-7f on Transcriptional Expression of Thyroid Differentiation Genes

Because RET/PTC-induced thyroid cell dedifferentiation is mediated by activation of the RAS-BRAF-MAPK pathway, the possible biologic significance of let-7f in TPC-1 cell differentiation was explored.

Transcription of specific molecular markers of thyroid differentiation such as thyroid transcription factor 1 (*TTF1*), thyroglobulin (*TG*), and sodium iodide symporter (*NIS*), was analyzed. let-7f induces a three-fold increase in *TTF1* mRNA expression and also a slightly increase in *TG* transcription when compared with control cells (Figure 5). However, *NIS* mRNA expression was not detected in TPC-1 cells even with let-7f overexpression (data not shown).

Discussion

Alterations in *RET*, *RAS*, and *BRAF* genes are major contributors to the transformation of normal thyroid cells to PTC [16]. Recent studies have associated these genetic alterations with global changes in the expression of miRNA [25–28]. This information has led toward new understanding of the genetic mechanisms and pathways underlying the etiopathogenesis of PTCs. Despite all the information concerning changes in the expression of miRNA in thyroid cancer, the functions of these molecules in thyroid tumorigenesis are poorly understood. In this study, we have identified a functional association of let-7f miRNA with thyroid cancer for the first time. The let-7 family of miRNA includes 14 isomers; each isomer is typically located on a different chromosome [8]. This study focused on let-7f, which is located at 9q22.3, because it has been previously shown to inhibit proliferation of lung cancer cells [29]. We observed a reduced expression of let-7f mature miRNA in PCCL3 cells with activation of *RET/PTC3*, but not *BRAF*. This suggests that let-7f down-regulation is an important step in the biologic effects mediated by the *RET/PTC* oncogene during malignant transformation. Supporting our data, a previous study comparing miRNA expression profile in thyroid tissues from patients with papillary carcinoma and normal thyroid tissues showed that let-7f is downregulated in this cancer [28]. To clarify the function of let-7f in the thyroid cancer scenery, exogenous let-7f was overexpressed in TPC-1 papillary cancer cells that harbor the *RET/PTC1* rearrangement. Although transfection of let-7f in TPC-1 cells repressed ERK phosphorylation, supporting the conclusion that RET/PTC-RAS-BRAF-ERK cascade activation is inhibited by this miRNA, significant modulation of K-RAS and H-RAS proteins was not observed after the induction of let-7f. Nevertheless, repression of MAPK signaling in concert with the modulation of genes related to cell cycle may explain, at least in part, the reduced cell growth of TPC-1 cells transfected with let-7f.

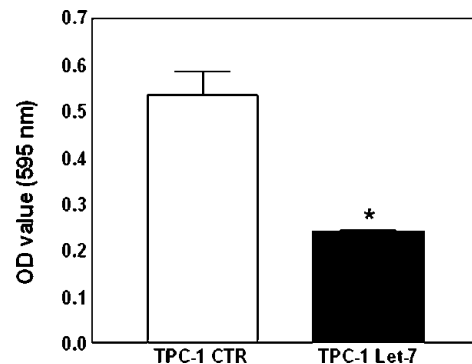


Figure 4. Effect of let-7 miRNA on the proliferation of TPC-1 cells. Cells were seeded at equal density and cultured until they reached semiconfluence. They were then incubated with MTT for 3 hours. Data are presented as the mean \pm SD of a single experiment performed in quintuplicate and are representative of two independent experiments. * $P = .000$ versus TPC-1 CTR.

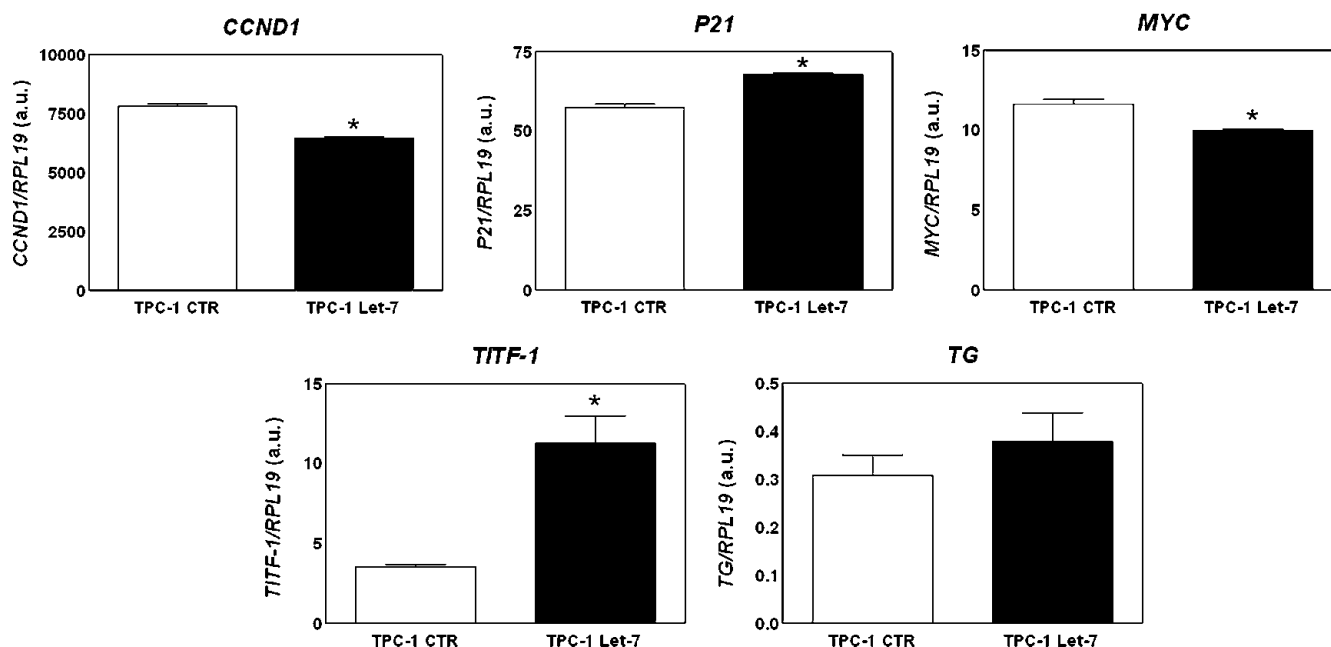


Figure 5. Effect of let-7 miRNA on gene expression. The graph shows the mRNA expression of the cell cycle-related genes *MYC*, *CCND1*, and *CDKN1A*, and the thyroid differentiation markers, *TTF1* and *TG*, as determined by quantitative RT-PCR. γ -Axis represents target gene expression normalized by *RPL19*. Columns in the graph represent mean \pm SD in arbitrary units (a.u.) of two independent reactions performed in triplicate. * $P = .000$ (*CCND1*), $.001$ (*P21*), $.006$ (*MYC*), $.001$ (*TTF-1*) versus TPC-1 CTR.

Previous studies from several groups have shown that constitutive activation of effectors along the RET/PTC-RAS-BRAF-ERK pathway impairs the expression of thyroid-specific genes, such as *TG*, *TPO*, *NIS*, and *TSHR*, and their corresponding transcription factors *TTF1* and *PAX8* [30–32]. During malignant progression, thyroid cancer cells undergo dedifferentiation, becoming more aggressive and refractory to treatment. We demonstrate here that let-7f is able to enhance the expression of some of these differentiation markers in TPC-1 cells, including *TTF1*, a major transcription factor in thyroid cells. *TTF1* is also key gene in thyroid development; mice lacking this transcription factor are unable to develop a thyroid gland [33]. The role of let-7 during development is well established, and it may also play an important role in thyroid development through the regulation of *TTF1* expression. Therefore, it is evident that let-7f is critical for proper regulation of thyroid cell growth and differentiation. It is known that let-7 has other targets in addition to *RAS* with respect to cancer, including the *HMG2* oncogene [34]. However, there is compelling evidence in the literature showing that activation of the MAPK signaling pathway is required for the development of RET/PTC-positive PTCs. Thus, the effects of let-7f in this specific cell (TPC-1) are likely due to its inhibitory effects on the MAPK pathway. Whether let-7f truly interacts with other mRNA that are important for its effects in TPC-1 cells is a question that remains to be answered. A few studies have previously associated alterations in miRNA with the development of PTCs, specifically using miRNA expression profiling to identify four miRNA (miR-146, miR-221, miR-222, and miR-181b) with high levels of expression in cancerous tissues [28,35]. In addition, it has been shown that the main genetic alterations observed in PTCs, the BRAF mutation and RET/PTC rearrangements, have global effects on the miRNA expression profile [26,27]. Along with these studies, our work demonstrates an association between this new class of small RNA and the development of thyroid carcinogenesis.

In summary, we show that reduced expression of let-7f is associated with RET/PTC malignant transformation. Furthermore, restoration of let-7f expression attenuates RET/PTC-mediated oncogenesis by preventing MAPK cascade activation and its resulting biologic effects, such as proliferation and dedifferentiation. Our data support a suppressor function of let-7f miRNA in thyroid cancer, revealing this molecule as a potential therapeutic target in patients with cancers harboring the RET/PTC fusion oncogene.

Acknowledgments

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Conflict of Interest Statement

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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