Thyroid Follicular Cell Loss of Differentiation Induced by MicroRNA miR-17-92 Cluster Is Attenuated by CRISPR/Cas9n Gene Silencing in Anaplastic Thyroid Cancer

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Background: Loss of the expression of thyroid differentiation markers such as sodium iodide symporter (NIS) and, consequently, radioiodine refractoriness is observed in aggressive papillary thyroid cancer and anaplastic thyroid cancer (ATC) that may harbor the BRAF^{V600E} mutation. Activation of the BRAF^{V600E} oncogene in thyroid follicular cells induces the expression of the *miR-17-92* cluster that comprises seven mature microRNAs (miRNAs). miRNAs are a class of endogenous small RNAs (~22 nt) that regulate gene expression post-transcriptionally. Indeed, *miR-17-92* is overexpressed in ATC, and *in silico* prediction shows the potential targeting of thyroid transcription factors and tumor suppressor pathways. In this study, we aimed to investigate the role of the *miR-17-92* cluster in thyroid cell differentiation and function.

Methods: *miR-17-92* silencing was performed using CRISPR/Cas9n-guided genomic editing of the *miR-17-92* gene in the KTC2 ATC cell line, and *miR-17-92* cluster or individual miRNAs were overexpressed in PCCl3 thyroid cells to evaluate the influence in thyroid cell differentiation and cell function.

Results: In this study, we demonstrate that CRISPR/Cas9n gene editing of the *miR-17-92* cluster results in promotion of thyroid follicular cell differentiation (NIS, thyroperoxidase, thyroglobulin, PAX8, and NKX2-1 expression) in the KTC2 ATC cell line and inhibits cell migration and proliferation by restoring transforming growth factor beta (TGF- β) signaling pathway responsiveness. Moreover, induction of the *miR-17-92* cluster in normal thyroid follicular cells strongly impairs thyroid differentiation and induces a pro-oncogenic effect by blocking TGF- β signaling and increasing cell migration.

Conclusions: *miR-17-92* is a potent regulator of thyroid follicular cell differentiation, and CRISPR/Cas9n-mediated editing of the *miR-17-92* gene efficiently blocks *miR-17-92* expression in the KTC2 ATC cell line, resulting in improvement of thyroid differentiation. Thus, targeting *miR-17-92* could provide a potential molecular approach to restoring thyroid cell differentiation and NIS expression in aggressive thyroid cancer.

Keywords: microRNAs, *miR-17-92*, thyroid cancer, differentiation, CRISPR/Cas9n

Introduction

THYROID CANCER IS the most frequent endocrine cancer and the most rapidly increasing cancer in incidence (1). Thyroid oncogenesis is mediated mainly by genetic alterations in MAPK signaling, with RET/papillary thyroid cancer (PTC) rearrangements, as well as RAS and BRAF mutations as the most prevalent alterations in PTC (2,3). Anaplastic thyroid cancer (ATC) is the most aggressive histotype of thyroid malignancy, and its pathogenesis remains incompletely un-

derstood. High-throughput next-generation sequencing data support a model of multistep carcinogenesis with ATC developing from well-differentiated tumors (4), and the main genetic alterations detected in ATC comprise *TP53*, *BRAF*, and *TERT* mutations (5–8). Nevertheless, most ATC cases are fatal due to the rapidly progressing tumors that become unresectable, refractory to radioiodine therapy, and that develop metastatic disease. Remarkably, recent studies described impressive results in the treatment of a BRAF^{V600E} advanced ATC with a neoadjuvant combined therapy of BRAF + MEK

inhibitors (dabrafenib + trametinib) and immunotherapy that turned the tumor resectable, and treatable with chemoradiation and sustained control with BRAF therapy in combination with immunotherapy (9,10). Indeed, the combined therapy of dabrafenib + trametinib has been recently approved by the Food and Drug Administration for the treatment of patients with locally advanced or metastatic ATC positive for the BRAF^{V600E} mutation and with no satisfactory locoregional treatment options.

MicroRNAs (miRNAs), small noncoding RNAs (~22 nt), have been recognized as potent regulators of gene expression; they target post-transcriptional regulation of messenger RNAs (mRNAs), modulating the vast majority of processes that contribute to tumorigenesis and progression. miRNA deregulation is one of the hallmarks of cancer (11), and high levels of the *miR-17-92* cluster have been detected in ATC (12). The *miR-17-92* cluster, also known as oncomiR-1, transcribes seven mature miRNAs (*miR-17-5p*, *miR-17-3p*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b*, and *miR-92a*) that ultimately exert a pro-oncogenic effect in various types of cancers (13–15). High-throughput miRNA expression studies have shown increased expression of *miR-17-92* in PTC (16), and our group showed that BRAF^{V600E} activates the *miR-17-92* cluster in normal thyroid follicular cells (17). The BRAF^{V600E} oncogene is associated with a loss of expression of the sodium iodide symporter (NIS/SLC5A5) and induces genomic instability in normal thyroid follicular cells (18), thus contributing to refractoriness to radioiodine therapy and disease progression, as observed in the aggressive subtypes of PTC and ATC (19). An *in silico* search showed that miRNAs can potentially target genes involved in thyroid cell differentiation (e.g., PAX8) and various components involved in thyroid hormone synthesis including NIS and thyroperoxidase (TPO) (20).

Investigating the role of *miR-17-92* could improve the understanding of thyroid cancer aggressiveness due to loss of differentiation. In this study, we demonstrate that CRISPR/Cas9n gene editing of the *miR-17-92* cluster results in promotion of thyroid follicular cell differentiation by increasing the expression of NIS, TPO, thyroglobulin (TG), PAX8, and NKX2-1 (previously known as TTF1) in the KTC2 ATC cell line, while inhibiting cell migration and proliferation by restoring transforming growth factor beta (TGF- β) signaling pathway responsiveness. Moreover, induction of the *miR-17-92* cluster in normal thyroid follicular cells strongly impaired thyroid differentiation and induced a pro-oncogenic effect. Thus, *miR-17-92* plays an important role in thyroid biology and differentiation, and modulating this cluster of miRNAs could improve thyroid cancer differentiation and cancer management.

Methods

Cell lines and culture

The human ATC cell line KTC2 was cultivated in RPMI supplemented with 5% fetal bovine serum (FBS). Stable KTC2 clones with *miR-17-92* cluster gene edited by CRISPR/Cas9n (named as KTC2-CI) were constructed according to the protocol of the laboratory of Ran *et al.* (21,22) by co-transfection of two plasmids containing guide RNAs (gRNAs) designed using the CRISPR Design website (<http://crispr.mit.edu>). The gRNAs, gRNA-A and gRNA-B, were designed to target two regions of the *miR-17-92* cluster in

MIR17HG (*miR-17-92* host gene) at chromosome 13 (Supplementary Table S1). The gRNA-A targets the region –24 nucleotides upstream of the precursor of pre-mir-17, and guide B targets the region +30 nucleotides inside pre-mir-17 (Fig. 1). The gRNA-A and gRNA-B were cloned at the *Bbs*I site downstream U6 promoter in the plasmids pSp-Cas9n(BB)-2A-PURO (PX462) and pSp-Cas9n(BB)-2A-GFP (PX461) (Addgene, Watertown, MA), respectively (Supplementary Table S2).

Genomic DNA was used to amplify an 800 bp target segment surrounding the gRNAs target site in the *miR-17-92* gene with specific primers (Surveyor primers) (Supplementary Table S1), and the Surveyor[®] Mutation Detection Kit (Integrated DNA Technologies, Skokie, IL) was used to confirm successful gene editing (mismatch) in the clones (Supplementary Fig. S1). GFP positive + puromycin-resistant single cell clones were expanded, and gDNA was extracted for sequencing confirmation after cloning the polymerase chain reaction (PCR) product into pGEM-T-easy (Promega, Madison, WI).

The rat PCC13 thyroid follicular cell line was cultivated in F-12 Coon's modified medium (Sigma, St. Louis, MO) supplemented with 5% FBS and four hormones and maintained at 37°C and 5% carbon dioxide (CO₂), as described previously (17). We transfected PCC13 cells with different plasmids (Supplementary Table S2) to generate stable cell lines with expression of the whole cluster (PCC13-miR-17-92), *miR-17* (PCC13-miR-17), *miR-19b* (PCC13-miR-19b), *miR-20a* (PCC13-miR-20a), *miR-92* (PCC13-miR-92a), and the segment with *miR-19a/miR-20a/miR-19b* (PCC13-miR-19a/miR-20a/miR-19b). As control, we transfected PCC13 cells with empty plasmid (PCC13- $\emptyset$).

Cell function assays

Cell counting. Growth curves were determined using KTC2 cells by seeding 2×10^4 cells per well in 6-well plates and cultivation for 24–72 hours. TGF- β 1 responsiveness was analyzed by seeding 5×10^4 KTC2 cells per well in 6-well plates. After 12 hours of adherence, cells received one pulse of recombinant TGF- β 1 (rTGF- β 1) (5 ng/mL) (rTGF- β 1; Peprotech, Rocky Hill, NJ) in the culture medium. Cells were detached by trypsinization after 48 hours and counted in a Guava minicytometer (Millipore, Burlington, MA). High-concentration iodine treatment in PCC13 cells was performed by diluting a 1 M stock solution of sodium iodide (NaI) in the medium to reach 10^{-3} M, and cell counting was performed after 24 hours of iodine treatment.

Cell viability assay (MTT). Cells were plated at 2×10^4 cells per well in 96-well plates and cultured for 24 hours. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cell culture medium to a final concentration of 250 μ g/mL and incubated at 37°C in 5% CO₂ incubator for 4 hours. Then, the medium was removed and formazan crystals were solubilized with 0.01 M HCl in isopropanol. Absorbance was measured at 595 nm in a plate spectrophotometer SpectraMax M (Molecular Devices, San Jose, CA).

Cell cycle. Cells were detached by trypsinization, pelleted with supernatant (dead cells), and fixed with 70% ethanol. After hydration in phosphate-buffered saline (PBS),

FIG. 1. CRISPR/Cas9n-edited *miR-17-92* gene in anaplastic thyroid cancer. (A) Result of genomic DNA sequencing of KTC2 clones 1 and 3 after CRISPR/Cas9n-mediated *miR-17-92* gene editing. sgRNA-guided target regions are highlighted in gray. Double nicking and DNA repair resulted in 15 nt deletion in clone 3 (Cl 3) and 4 nt deletion +4 nt changes in clone 1 (Cl 1) *miR-17-92* gene. (B) Gene expression validation of *miR-17-92* mature miRNAs levels in CRISPR/Cas9n-edited KTC2-Cl1 and KTC2-Cl3. Data are expressed as mean $\pm$ SD ($n=3$) for gene expression. *** $p < 0.0001$ versus KTC2-Wt. miRNAs, microRNAs; SD, standard deviation; sgRNA, single guide RNA.

cells were treated with RNase (100 μ g/mL) and DNA was stained with propidium iodide (50 μ g/mL). The cell cycle was characterized by counting 10,000 events under flow cytometry using Guava EasyCyte Mini (Millipore).

Wound assay. 1×10^5 cells were plated in 6-well plates and cultured until confluency. Next, 2 μ g/mL mitomycin (Sigma) was added to the medium for 2 hours before performing the scratch in the monolayer using a 100- μ L tip. After several washes with PBS, the medium was replaced by complete medium containing 2 μ g/mL mitomycin (Sigma). Wound closure was monitored until cells completely repopulated the scratch (48 hours) under an inverted micro-

scope EVOS XL Core Imaging System (Thermo Fisher Scientific, Waltham, MA).

Transwell assay. A migration assay was performed using uncoated TranswellTM chambers with membrane pore size of 8.0 μ m (Corning Inc, Corning, NY). 2×10^4 cells were suspended in culture medium containing 0.5% FBS and plated in the upper chamber, whereas the lower chamber contained culture medium with 5% FBS. The cells that migrated through the membrane within 24 hours were fixed in 4% paraformaldehyde diluted in PBS and stained with 0.5% crystal violet. Cells were photographed using a Nikon Eclipse

E600 microscope equipped with a CF160 epi-fluorescence optical camera and counted (10 representative fields).

Gene expression by quantitative PCR

Total RNA was extracted using the phenol–chloroform method using the TRIzol reagent (Invitrogen-Thermo Fisher, Carlsbad, CA). Protein coding gene expression was investigated using complementary DNA (cDNA) generated from the reverse transcription of 1 μ g of total RNA using oligo-dT primer and MMLV reverse transcriptase (Invitrogen-Thermo Fisher). Quantitative PCR was performed in a ViiA7[®] Sequence Detection System (Applied Biosystems-Thermo Fisher, Foster City, CA) using SYBR Green Master Mix, cDNA, and specific primers (Supplementary Table S1). Gene expression was normalized by comparison with *Rpl19* levels and calculated using the QGene program (23).

For miRNA expression, 10 ng of total RNA was reverse transcribed using a TaqMan[®] Reverse Transcription Kit (Applied Biosystems-Thermo Fisher) in the presence of stem-loop primers, followed by quantitative PCR using TaqMan MicroRNA Assays for *miR-17-5p* (assay 393), *miR-18a-5p* (assay 2422), *miR-19a-3p* (assay 395), *miR-19b-3p* (assay 396), *miR-20a-5p* (assay 580), *miR-92a-3p* (assay 430), small nucleolar RNA (*snoRNA*) (assay 1718), and *RNU6B* (assay 1093) (Applied Biosystems-Thermo Fisher), and TaqMan Universal PCR Master Mix, No AmpErase[®] UNG (Life Technologies-Thermo Fisher, Carlsbad, CA) in a ViiA7 Sequence Detection System. Gene expression was normalized by comparison to *snoRNA* levels and calculated using the QGene program (23).

Western blotting analyses

Total protein was extracted from cells using 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). Protein concentration was determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA), and 40 μ g of each sample was fractionated by 10% SDS-PAGE and blotted onto a nitrocellulose Hybond-ECL membrane (Amersham Biosciences, Little Chalfont, UK). Nonspecific binding sites were blocked with 5% nonfat dry milk in Tris-buffered saline-0.1% Tween-20. The following primary antibodies were used: mouse anti-SMAD4 (sc-7866), rabbit anti-TGFBRII (sc-220), rabbit anti-TTF1 (sc-13040), mouse anti-PAX8 (sc-81353), rabbit anti-cMYC (sc-764), rabbit anti-pSMAD3 (sc-130218), mouse anti- α -tubulin (sc-5286), mouse anti- β -actin (sc-47778), mouse anti-vimentin (sc-32322) (Santa Cruz, Santa Cruz, CA), and rabbit anti-LIN28B (Cell Signaling, Danvers, MA). The rabbit anti-NIS antibody was kindly donated by Dr. Sissy Jhiang. Antibody bound to target protein was detected with horseradish peroxidase-conjugated secondary antibodies and developed with luminol and p-coumaric acid (Sigma) reagents in the presence of hydrogen peroxide. Chemiluminescence emission was visualized with a ImageQuant LAS4000 imaging system (GE Healthcare, Little Chalfont, UK).

Bioinformatics and luciferase assay

miR-17 and miR-20 target validation. A segment of the rat *Pax8* and human *PAX8* 3'-untranslated region (UTR)

containing the predicted site *miR-17/miR-20* (position 1079–1085 in rat and position 1990–1997 in human mRNA) extracted from TargetScan database (24) was cloned into pmiR-Glo plasmids (Promega) according to the manufacturer's instructions. Briefly, to generate the wild-type (Wt) *miR-17/20* binding site reporter plasmid (pmiR-Glo-Pax8-Wt), two oligonucleotides were annealed and ligated in pmiR-Glo digested with *XhoI* and *XbaI*. As control, we also generated mutated plasmids that contained mutations in the seed sequence of miRNA binding sites (pmiR-Glo-Pax8-mut) that would block miRNA interaction. We also constructed a *miR-19* and a *miR-17/20* pmiR-Glo plasmid to test *DICER1* as potential target (Supplementary Table S1). Transient transfection of pmiR-Glo-Pax8-Wt and pmiR-Glo-Pax8-mut was performed in PCCI-miR-17, PCCI3-miR-20a, and PCCI3-miR-19b cells. Forty-eight hours after transfection, cell lysates were collected and firefly luciferase activity was measured in a SpectraMax L Luminometer (Molecular Devices) using a Dual Luciferase[®] Reporter assay system (Promega). Luminescence was normalized by *Renilla* luciferase levels.

Nis, Tg, and Tpo promoter activity. For this luciferase reporter assay, we used the following plasmids: pNIS-2.8 (25) plasmid contains the 2854-bp fragment of the rat *Nis* promoter; pGL-Tg (26) contains a 202-bp fragment of the human *TG* promoter, and pTPO-luc contains a 420-bp fragment of the human *TPO* promoter (27). Three hundred nanograms of pNIS 2.8, pGL-Tg, or pTPO-luc plasmids was co-transfected with 30 ng of *Renilla* pRL plasmid as control for transfection efficiency using Lipofectamine 3000 (Life Technologies-Thermo Fisher). Cell lysates were collected after 24 hours of transfection. Firefly and *Renilla* luciferase were measured using the Dual-Luciferase Reporter assay system (Promega) in GloMax[®] 20/20 Luminometer (Promega).

TGF- β signaling activity. To evaluate the responsiveness to TGF- β signaling, we used the SBE4 Luc plasmid that contains four copies of the *Smad* Binding Element (SBE) or a p3TP-lux plasmid (Addgene) (Supplementary Table S2). Briefly, 4×10^4 cells were seeded into 24-well plates and transfected with 300 ng of SBE4-Luc (or p3TP-lux) plus 30 ng of pRL (*Renilla* luciferase) using Lipofectamine 2000 (Invitrogen-Thermo Fisher) before the treatment with 1 ng/mL (20 U/mL) rTGF- β (Peprotech) in culture medium for 24 hours. Cell lysates were collected after 24 hours, and luminescence was measured as described previously. To evaluate the effect of *TGFB2* knockdown on TGF- β signaling, we transiently transfected KTC2 cells with a SGEP plasmid containing short hairpin RNA for human *TGFB2* mRNA (Supplementary Table S1) 48 hours before transfection with TGF- β reporter plasmid SBE4 luc. Then, cells were treated with 1 ng/mL TGF- β 1 for 24 hours, and cell lysates were collected and luminescence was measured as previously described.

Statistical analysis

The results are presented as the mean $\pm$ standard deviation, and they were submitted to analysis of variance followed by the Tukey test. Differences were considered significant at $p < 0.05$.

Results

miR-17-92 silencing via CRISPR/Cas9n induces thyroid follicular cell differentiation and restores TGF- β antimitogenic response

As *miR-17-92* cluster expression is increased in ATC samples (12) and cell lines (Supplementary Fig. S2), we investigated the role of silencing *miR-17-92* in thyroid cell function. To that end, we used the CRISPR/Cas9n system to efficiently edit the *miR-17-92* gene in the human ATC cell line KTC2 and knock down all the cluster components simultaneously. Using two CRISPR/Cas9n plasmids, we designed two single guide RNAs to target the 5' region upstream of the *miR-17* precursor. This region contains the splicing factor binding site (Fig. 1A) essential for primary *miR-17-92* processing into mature miRNAs (28). The edition of the *miR-17-92* gene was efficient in ATC cell line KTC2 as observed in genomic DNA sequencing of two clones (KTC2-C11 and KTC2-C13) (Fig. 1A). The gene editing resulted in global downregulation of *miR-17-92* mature miRNA levels, ranging from >90% inhibition of *miR-17-5p* to >40% inhibition for *miR-92a* (Fig. 1B).

After this initial validation of the system, we evaluated expression of thyroid differentiation genes in response to *miR-17-92* silencing by CRISPR/Cas9n. We observed an increase in the gene expression of *NIS*, *TPO*, *TG*, and the transcription factors *PAX8* and *NKX2-1* in KTC2-C11 and KTC2-C13 (Fig. 2A). *NIS* mRNA expression was detected at very low levels that increased after *miR-17-92* silencing. Accordingly, protein expression of *PAX8* and *NKX2-1* was increased in KTC2-C11 and KTC2-C13 analyzed by Western blot (WB) (Fig. 2B). However, *NIS* and *TPO* protein were undetectable by WB and immunofluorescence (data not shown).

Next, we used the luciferase reporter plasmids pNis 2.8, pGL-Tg, and Tpo-luc to investigate promoter activity of the *NIS* (*SLC5A5*), *TG*, and *TPO* genes, respectively. We observed a strong induction of *TG* and *TPO* promoters in *miR-17-92* CRISPR/Cas9n-edited KTC2-C11 and KTC2-C13, while interestingly *NIS* gene promoter activity was reduced compared with KTC2-Wt cells (Fig. 2C).

Functionally, targeting *miR-17-92* by CRISPR/Cas9n exerted an antitumorigenic effect in KTC2 cells. We observed a reduced cell count of KTC2-C11 and KTC2-C13 clones compared with KTC2-Wt cells (Fig. 3A). Moreover, cell colony formation (Fig. 3B) and cell migration (in the presence of mitomycin) (Fig. 3C) were strongly repressed in KTC2-C11 and KTC2-C13 clones, leading us to investigate TGF- β signaling, an important antiproliferative pathway for thyroid cells. We observed increased protein levels of the TGF- β signaling components TGFBR2 and SMAD4 (Fig. 3D), both validated targets of *miR-17-92* (17,29), in KTC2-C11 and KTC2-C13 clones compared with KTC2-Wt cells. Interestingly, MYC protein levels were reduced in KTC2-C11 and KTC2-C13 clones (Fig. 3D). MYC is a known positive regulator of *miR-17-92* (30) and is repressed by TGF- β signaling activation (31).

To evaluate whether TGF- β signaling responsiveness was altered in KTC2 clones after *miR-17-92* edition, we performed cell counting in the presence of recombinant active TGF- β 1 and observed that KTC2-C11 and KTC2-C13 showed recovery of the antimitogenic response to TGF- β 1 treatment leading to reduction in cell number, an effect not observed in

KTC2-Wt cells (Fig. 3E). Indeed, KTC2-C11 and KTC2-C13 showed high basal levels of pSMAD3 nuclear staining compared with KTC2-Wt indicating a reactivation of TGF- β signaling in these cells (Fig. 3F). This finding was further confirmed by a SBE4 luciferase reporter assay that showed a 60% and 80% higher response after recombinant TGF- β 1 treatment in KTC2-C11 and KTC2-C13, respectively, compared with KTC2-Wt cells (Fig. 3G).

miR-17-92 overexpression impairs TGF- β signaling in normal thyroid follicular cells

To investigate the role of *miR-17-92* in normal thyroid cell biology, we overexpressed the *miR-17-92* cluster in normal rat thyroid follicular cells PCC13 (stable cell line PCC13-miR-17-92) (Fig. 4A). *miR-17-92* overexpression increased cell viability measured by MTT (Fig. 4B) and resulted in a fourfold increase in cell migration in PCC13-miR-17-92 cells evaluated by a transwell assay (Fig. 4C), although the cell count was not significantly altered (data not shown).

As *miR-17-92* targets are enriched in the TGF- β signaling pathway, we analyzed the protein levels of TGFBR2 and SMAD4 and observed a reduction of TGFBR2 in PCC13-miR-17-92 (Fig. 4D). To investigate whether TGFBR2 reduction would impact TGF- β signaling, we used a 3TP-lux luciferase reporter assay to evaluate TGF- β responsiveness. We observed that the treatment of control PCC13- $\emptyset$ cells with recombinant active TGF- β 1 resulted in a dose-dependent increment in luciferase reporter activity (Fig. 4E). However, this effect was significantly reduced by *miR-17-92* overexpression in PCC13-miR-17-92 cells (Fig. 4F). Moreover, TGF- β 1 treatment resulted in a smaller reduction in cell counting for PCC13-miR-17-92 cells compared with control PCC13- $\emptyset$ (Fig. 4F). The analysis of the cell cycle showed that TGF- β 1 treatment in PCC13-miR-17-92 cells does not induce G1-cell cycle arrest as observed in PCC13- $\emptyset$ cells (Fig. 4G).

To further evaluate the role of *miR-17-92* in TGF- β signaling, we next tested the effect of a high iodine concentration on the cell count of PCC13-miR-17-92 cells. High iodine induces an antiproliferative effect that is dependent on the activation of the TGF- β pathway (32). We observed that high iodine concentration (10^{-3} M NaI) decreased the cell count in control cells (PCC13- $\emptyset$), but this effect was attenuated after overexpression of *miR-17-92* in PCC13-miR-17-92 cells (Fig. 4H). Next, we investigated the influence of *NIS* in this effect as this protein is essential to iodine trapping in thyroid follicular cells. Surprisingly, Western blotting showed that PCC13-miR-17-92 cell exhibited reduced *NIS* levels (Fig. 4I) that could contribute to the refractoriness to high iodine treatment. Altogether, these results show that *miR-17-92* activation in normal thyroid follicular cells exerts a pro-proliferative effect by blunting TGF- β signaling responsiveness.

miR-17-92 overexpression impairs thyroid follicular cell differentiation in normal thyroid follicular cells

The expression analysis of genes involved in thyroid differentiation showed that overexpression of *miR-17-92* severely repressed *Nis* and *Tpo* gene expression, which are related to iodine metabolism, as well as the thyroid transcription factors *Nkx2-1* and *Pax8* in PCC13-miR-17-92 cells (Fig. 5A). The analysis of *Nis* (*Slc5a5*) and *Tg* promoter activity using a luciferase reporter assay showed that the *Nis*

FIG. 2. Effect of CRISPR/Cas9n editing of *mir-17* gene in thyroid cell differentiation. (A) Gene expression of thyroid differentiation genes *NIS*, *TPO*, *TG*, and transcription factors *PAX8* and *NKX2-1* in KTC2-Cl1 and KTC2-Cl3 compared with KTC2-Wt. Data are expressed as mean $\pm$ SD ($n = 3$) for gene expression. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus KTC2-Wt. (B) Protein levels of *PAX8* and *NKX2-1* transcription factors by WB in KTC2-Cl1 and KTC2-Cl3 compared with KTC2-Wt. (C) *NIS*, *TPO*, and *TG* genes promoter activity using luciferase reporters pNIS2.8, pTPO, and pGL-TG. Representative results of three independent experiments. Data are expressed as mean $\pm$ SD ($n = 4$). * $p < 0.05$, *** $p < 0.001$ versus KTC2-Wt. a.u., arbitrary units; TG, thyroglobulin; TPO, thyroperoxidase; WB, Western blot.

FIG. 3. Effect of *miR-17-92* gene editing in KTC2 thyroid cell biology. (A) Cell counting of KTC2-C11 and KTC2-C13 compared with KTC2-Wt. $^{**}p < 0.05$, $^{***}p < 0.01$ versus KTC2-Wt. (B) Clonogenic assay of KTC2-C11 and KTC2-C13 compared with KTC2-Wt after 8 days of cells seeding. Colonies were stained with crystal violet. Representative results of three independent experiments. (C) Wound assay was performed for KTC2-C11 and KTC2-C13 compared with KTC2-Wt in the presence of 2 μ g/mL mitomycin, and images were taken 48 hours after scratch. (D) Protein levels of TGF- β signaling components SMAD4 and TGFB2, and cMYC. (E) Analysis of responsiveness to TGF- β 1. Cell counting was performed in KTC2-C11 and KTC2-C13 compared with KTC2-Wt after 48 hours of treatment with rTGF- β 1 at 5 ng/mL. $^{*}p < 0.05$, $^{***}p < 0.01$. (F) Immunofluorescence for pSMAD3 in KTC2-Wt and KTC2-C13 compared with KTC2-Wt after 48 hours of treatment with rTGF- β 1 (Alexa 488), while the nuclei are stained in blue (DAPI). (G) Luciferase reporter assay (SBE4-Luc) to measure activation of TGF- β signaling in KTC2-C11 and KTC2-C13 compared with KTC2-Wt in the presence of 1 ng/mL TGF- β 1, or (H) when TGFB2 is knocked down by shRNA and cells are treated with 1 ng/mL TGF- β 1. $^{*}p < 0.05$, $^{***}p < 0.01$, $^{***}p < 0.001$. DAPI, 4',6-Diamidino-2-phenylindole dihydrochloride; shRNA, short hairpin RNA; TGF- β , transforming growth factor beta; rTGF- β 1, recombinant TGF- β 1.

FIG. 4. Effects of *miR-17-92* overexpression in normal rat thyroid follicular cells PCC13. (A) Validation of *miR-17-92* overexpression in PCC13-miR-17-92 by measuring *miR-19a* and *miR-19b* mature miRNA levels. (B) *miR-17-92* overexpression increases cell viability measured by MTT assay in PCC13-miR-17-92 compared with PCC13- ϕ . (C) Induces cell migration in a transwell assay. * $p < 0.05$, *** $p < 0.001$ versus PCC13- ϕ . Representative results of two independent experiments. Data are expressed as mean $\pm$ SD ($n = 3$). (D) Protein levels of TGFBR2 and SMAD4 in PCC13-miR-17-92 compared with PCC13- ϕ by WB. Overexpression of *miR-17-92* in PCC13 reduces responsiveness to exogenous TGF- β treatment. (E) Luciferase assay with 3TP-lux plasmid in the presence of 1 or 2 ng/mL TGF- β 1 in PCC13-miR-17-92 compared with PCC13- ϕ . (F) Cell counting and (G) cell cycle analysis 24 hours after 1 ng/mL TGF- β 1 treatment in PCC13-miR-17-92 compared with PCC13- ϕ . Representative results of two independent experiments in triplicate. (H) High-iodine concentration (10^{-3} M) effect in PCC13-miR-17-92 cells counting after 48 hours of treatment compared with PCC13- ϕ . (I) Nis protein expression during high-iodine treatment (10^{-3} M NaI) in PCC13-miR-17-92 compared with PCC13- ϕ . * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are expressed as mean $\pm$ SD ($n = 3$). MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaI, sodium iodide.

and *Tg* genes are transcriptionally repressed after *miR-17-92* overexpression in PCC13 cells (Fig. 5B). At the protein level, we also observed an important reduction in NIS and PAX8, while no modulation was observed in NKX2-1. Moreover, *miR-17-92* induced the expression of vimentin and LIN28B, markers for mesenchymal cells and stem cells, respectively (Fig. 5C).

miR-17-92 cluster components modulate thyroid differentiation

To dissociate the role of individual miRNAs that comprises the *miR-17-92* cluster, we transfected PCC13 cells with plasmids to stably express *miR-17*, *miR-19b*, *miR-20a*, *miR-92a* individually and the *miR-19a/miR-20a/miR-19b* cluster

FIG. 5. Overexpression of *miR-17-92* cluster reduces differentiation genes in normal rat thyroid follicular cells. (A) *miR-17-92* inhibits thyroid differentiation genes and transcription factors expression in PCCl3-miR-17-92 compared with PCCl3-φ. (B) *Nis* and *Tg* promoter activity is reduced when *miR-17-92* is overexpressed in PCCl3-miR-17-92. * $p < 0.05$, ** $p < 0.01$ versus PCCl3-φ. Representative results of two independent experiments. Data are expressed as mean $\pm$ SD ($n = 3$). (C) *miR-17-92* overexpression reduces protein levels of NIS and PAX8, while increases stem cell markers LIN28B expression, and Vimentin.

FIG. 6. Overexpression of *miR-17-92* cluster components reduces differentiation genes in normal rat thyroid follicular cells. (A) Validation of *miR-17-92* cluster components overexpression by qPCR. (B) Protein expression of NIS, PAX8, and NKX2-1 analyzed by WB in PCC13 overexpressing *miR-17-92* components. (C) *Nis* and *Tg* promoter activity upon *miR-17-92* components overexpression. PCC13 cells were transfected with pNIS 2.8 reporter plasmid or pGL-Tg plasmid, and cell lysates were collected after 24 hours. Luciferase levels were normalized by the co-transfection of pRL plasmid that expresses Renilla luciferase gene. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus PCC13- ϕ . Representative results of two independent experiments. Data are expressed as mean $\pm$ SD ($n = 4$). qPCR, quantitative polymerase chain reaction; snoRNA, small nucleolar RNA.

as a group (Fig. 6A). We observed that individually *miR-19b*, *miR-92a*, and *miR-17* strongly inhibited the expression of NIS, while *miR-20a* showed no effect on NIS protein expression (Fig. 6B). Indeed, the combined effect of the whole *miR-17-92* cluster components in PCC13-miR-17-92 resulted in NIS downregulation (Fig. 5C). For the thyroid transcription factor PAX8, we observed that individually *miR-17*, *miR-19b*, and *miR-20a* inhibited PAX8 protein levels (Fig. 6B) resulting in PAX8 inhibition when the whole *miR-17-92* cluster is overexpressed (Fig. 5C) in PCC13 cells. However, the expression of individual or combined *miR-17-92* components did not change NKX2-1 protein levels (Figs. 5C and 6B).

In this context, deregulation of thyroid transcription factor levels could impair NIS protein by influencing the transcriptional activity of the *Nis* gene. To investigate this possibility, we used the reporter plasmid pNIS 2.8 as it contains a 2.8-Kb fragment of the rat *Nis* gene promoter comprising two PAX8 and one NKX2-1 site (25). Indeed, the activation of the *miR-17-92* cluster components lead to transcriptional im-

pairment of the *Nis* gene and a reduction in NIS levels in normal thyroid cells PCC13 (Fig. 6C). *miR-19* expression *per se* or in combination (*miR-19a/miR-20a/miR-19b*), and *miR-17* exerted important repressive effects on *Nis* gene transcription. For *miR-20a*, we did not observe any significant modulation of *Nis* gene transcription, a result that was confirmed by analyzing NIS expression by WB. Interestingly, *miR-92a* slightly induced *Nis* transcriptional activity (Fig. 6C). We observed similar results when analyzing *Tg* promoter activity using a pGL-Tg reporter plasmid in PCC13 cells, showing transcriptional inhibition when *miR-17*, *miR-19* *per se*, or in combination (*miR-19a/miR-20a/miR-19b*) were overexpressed (Fig. 6C). Overall, the expression of the whole *miR-17-92* cluster components leads to transcriptional inhibition of the *Nis* and *Tg* promoters in normal thyroid follicular cells PCC13 (Fig. 5B).

These results indicate that *miR-17-92* exerts its effects on thyroid differentiation through a common mechanism mediated by thyroid transcription factors. Consequently, we used an *in silico* approach to search for *miR-17-92* cluster

targets relevant to thyroid cell function. Indeed, analysis with the TargetScan tool revealed that the 3'-UTR segment of rat *Pax8* mRNA contains predicted sites for *miR-17-5p* and *miR-20a-5p*. To test the interaction of these miRNAs and *Pax8* 3'-UTR, we constructed a reporter plasmid that contained the Wt sequence of the rat *Pax8* 3'-UTR (position 1079–1085 in the 3'-UTR sequence) predicted to interact with *miR-17-5p* and *miR-20a-5p*, cloned downstream of Luciferase gene in pmiR-Glo plasmid. We also constructed a mutated plasmid (mut) containing mismatches in the region of miRNA/mRNA pairing as control. We transfected these plasmids into PCC13 cells overexpressing single components of the cluster, *miR-17*, *miR-20a*, and *miR-19b* (specificity control) and did not observe a significant downregulation of luciferase activity neither in *miR-17*-expressing cells nor in *miR-20a*-expressing cells (data not shown). This result indicates that in this model *Pax8* mRNA is not a target for *miR-17-5p/miR-20a-5p*.

Moreover, studies using different mouse models for *Dicer1*-specific knockout in thyroid follicular cells have shown that miRNAs are necessary to maintain thyroid cell differentiation and function. In these models, loss of *DICER1* leads to severe hypothyroidism, and a loss of thyroid cell differentiation and proliferation (33,34). The *in silico* search also showed that rat *Dicer1* mRNA 3'-UTR contains one predicted site for an interaction with *miR-17-5p/miR-20-5p* and another conserved site for *miR-19-3p*. We also constructed a pmiR-Glo plasmid containing the interaction site for *miR-17-5p/miR-20a-5p* (position 2722–2728) and *miR-19-3p* (position 1324–1330). However, our results did not show any significant modulation of these *Dicer1* 3'-UTR luciferase reporters in response to the modulation of *miR-17-5p/miR-20a-5p* and *miR-19-3p* (data not shown), indicating that in this model, *Dicer1* mRNA is not a target for these miRNAs.

Discussion

Overexpression of *miR-17-92* is detected in several types of cancers as an important marker of malignancy and tumor aggressiveness (13,35). Indeed, high levels of *miR-17-92* are detected in aggressive ATC (12). We show in this study that CRISPR/Cas9n-mediated editing of the *miR-17-92* gene is efficient and results in a strong inhibition of *miR-17-92* mature miRNAs in the ATC cell line. We designed the gRNAs to target the upstream region of *miR-17* pre-miR that contains a splicing site essential for *pri-mir-17-92* processing (28). Importantly, we observed a recovery of the expression of the thyroid-specific genes *TPO* and *TG*, and to some extent *NIS*, as well as the *PAX8* and *NKX2-1* transcription factors. Re-establishing *NIS* expression is one of the biggest challenges of treating aggressive thyroid cancers. *NIS* suppression in thyroid cancer is multifactorial and related to epigenetics, transcriptional, and post-transcriptional regulation that involves DNA methylation, MAPK signaling, and miRNAs (18,36,37).

The loss of expression of thyroid differentiation markers related to iodine metabolism is a key event during thyroid oncogenesis and is correlated with aggressive tumor behavior, such as radioiodine refractoriness (RAIR). Ultimately, RAIR is caused by the loss of the iodine-trapping ability mediated by *NIS* downregulation. Indeed, RAIR thyroid tumors display a high frequency of *BRAF* mutation and alter-

ations in the RAS and PI3K signaling pathways (19). Activation of the *BRAF*^{V600E} oncogene is a well-known inhibitor of *NIS* and thyroid cell differentiation (18). Our group showed recently that the *BRAF*^{V600E} mutation increases *miR-17-92* expression in normal thyroid follicular cells and reduces TGF- β signaling responsiveness (17). Moreover, the *BRAF*^{V600E} oncogene can be associated with aggressive clinical-pathological characteristics, such as extrathyroidal invasion, and mortality in thyroid cancers (38–40).

In our study, we show that the activation of the *miR-17-92* cluster is an important step to downregulate *NIS* and thyroid cell differentiation, leading to a loss of iodide uptake *in vitro* and, hence, expected to be associated with refractoriness to radioiodine *in vivo*. We dissected the role of individual miRNA components of the *miR-17-92* cluster in thyroid differentiation, especially the impact on *NIS* levels, and observed that while some miRNAs such as *miR-17-5p*, *miR-19a/b*, and *miR-92* may inhibit *NIS* expression, others such as *miR-20a-5p* do not modulate *NIS* levels. Moreover, *miR-17*, *miR-19*, and *miR-20a* downregulate the *PAX8* transcription factor, indicating that a cooperative targeting of thyroid differentiation genes and transcription factors by the *miR-17-92* cluster is a key effect of this oncomiR in thyroid cancer. Taken together, the single components of the *miR-17-92* cluster have inhibitory and stimulatory effects in thyroid differentiation, but the overexpression of the whole *miR-17-92* cluster results in an overall inhibitory effect in thyroid follicular cells.

Recently, other miRNAs have been implicated in the regulation of *NIS* and thyroid differentiation such as *miR-339-5p* and *miR-146b-3p* (41,42). *miR-339-5p* is increased in human PTC and targets the 3'-UTR of *NIS* mRNA, and its overexpression efficiently reduces *NIS* mRNA levels and radioiodine trapping in PCC13 and MCF7 cells (41). Moreover, *miR-146b-5p* is the most overexpressed miRNA in PTC and a hallmark of thyroid cancer, and the 3p strand (*miR-146b-3p*) mediates the targeting of *NIS* and *PAX8* mRNAs, leading to the downregulation of thyroid differentiation (42).

In this study, we also show that *miR-17-92* activation leads to a loss of responsiveness to the antimitogenic effect of TGF- β in normal thyroid cells via deregulation of TGF- β signaling components, such as TGFBR2 and SMAD4. In a previous study, we showed that *BRAF*^{V600E} oncogene induces *miR-19a/b* that targets *SMAD4* mRNA (17), an effect also observed in *SMAD4* when we overexpressed *miR-19b* in PCC13 cells (data not shown).

Moreover, silencing of the mature *miR-17-92* in KTC2 cells using CRISPR/Cas9n reduced cell counts and increased SMAD4 and TGFBR2 levels, known targets of *miR-17* and *miR-19* (29), which led to partial restoration of the antimitogenic effect upon rTGF- β 1 treatment. Interestingly, cMYC protein levels were reduced after *miR-17-92* gene silencing. cMYC is one of the transcriptional regulators of *miR-17-92* (30), and studies have shown that *miR-17-92* cooperates with/mediates MYC effects to promote tumorigenesis (14,43). However, reduction of MYC levels could be associated with the reactivation of TGF- β signaling, which has been shown to repress cMYC transcription (31).

In this study, we found a pro-oncogenic role of the *miR-17-92* cluster associated with a loss of differentiation, a loss of *NIS* expression, and refractoriness to the antiproliferative signal of TGF- β . By dissecting the cluster into separate

miRNAs, we demonstrate that *miR-19*, *miR-92*, and *miR-17* exert important repressive effects on NIS, NKX2-1, and PAX8 protein levels. Targeting *miR-17-92* via CRISPR/Cas9n gene editing resulted in an effective and stable reduction of mature *miR-17-92* and induced a partial restoration of a differentiated phenotype characterized mainly by reestablishing expression of NIS, TG, TPO, PAX8, and NKX2-1. Moreover, it restored TGF- β signaling responsiveness in an ATC cell line. The success of CRISPR/Cas9 editing to correct diseases in animal models and its recent success in human embryos (44–46) may ultimately open new perspectives for patients with incurable or aggressive diseases, such as cancer.

Conclusions

miR-17-92 is a potent regulator of thyroid follicular cell differentiation, and CRISPR/Cas9n-mediated gene editing is efficient to silence *miR-17-92* expression in an ATC cell line, resulting in improvement of thyroid differentiation and TGF- β antiproliferative response while inducing antitumorigenic effects. Thus, molecular targeting of *miR-17-92* could be a potential therapeutic approach for aggressive thyroid carcinomas.

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Author Disclosure Statement

The authors declare that there is no conflict of interest that would impair the impartiality of this article.

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Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Table S1
Supplementary Table S2

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