MicroRNA-7 directly targets *Reg1* in pancreatic cells

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List of abbreviations

3'UTR: 3' untranslated region

AD: Alzheimer disease

EGFR: Epidermal growth factor receptor

hfPCR: high fidelity polymerase chain reaction

PanIN: pancreatic intraepithelial neoplasia

PDAC: pancreatic ductal adenocarcinoma

PMSF: phenylmethanesulfonyl fluoride

PVDF: polyvinylidene difluoride

Abstract

Regenerating islet-derived (Reg) proteins, which were first discovered in the pancreas, are associated with increased proliferation, prevention of apoptosis and enhanced differentiation in normal and disease states but very little is known about the regulation of their expression. We hypothesized that Reg expression is influenced by microRNAs. Bioinformatic analysis predicted Reg1 to be a target of microRNA-7 (miR-7), which influences pancreatic beta-cell function. To this end, we investigated the effects of miR-7 on Reg1 expression in pancreatic acinar and islet beta-cells. High levels of Reg1 were noted by immunostaining and western blotting in acinar cells in contrast to islet cells. A reciprocal expression pattern was observed for miR-7. Overexpression of miR-7 resulted in Reg1 mRNA suppression and reduction of secreted Reg1 protein. Conversely, miR-7 knockdown led to increases in Reg1. Targeting of Reg1 by miR-7 was confirmed via luciferase activity assays. In contrast, miR-7 did not directly repress the human ortholog of Reg1, REG1A, as well as REG1B indicating species differences in the regulation of Reg expression. This is the first account of microRNA modulation of any Reg member warranting studies to fill gaps in our knowledge of Reg protein biology, particularly in disease contexts.

Keywords: Reg proteins; microRNA-7; pancreas; islets; acinar cells.

Introduction

Regenerating islet-derived (Reg) proteins are C-type lectin-like proteins originally discovered in patients with chronic calcific pancreatitis (10) while subsequent studies linked Regs further to pancreatitis (5, 22, 32) but also to pancreatic cancer and diabetes. In particular, murine Reg1 and its human ortholog REG1A (also known as lithostathine) are expressed in the exocrine pancreas and hyperplastic islets but not in normal islets. Transgenic mice expressing Reg1 from a glucagon promoter in their islets exhibit apoptosis of β-cells with ensuing diabetes and develop malignant tumors (48). Yet, Reg1 helps restore β-cell mass after pancreatectomy in rats (43) and prevents or delays the development of diabetes in non-obese diabetic mice (44). Elevated plasma Reg1 has been observed in mice with pancreatic intraepithelial neoplasia (PanIN) lesions (16), while REG1A and REG1B are detected in the serum and urine of pancreatic ductal adenocarcinoma (PDAC) patients (30, 39). Reg1 has also been implicated in extrapancreatic pathologies including colon cancer (49), seminoma (33), hepatocellular carcinoma (8), cholangiocarcinoma (21) and Alzheimer disease (AD) (11, 12).

Despite the protein's significant presence in various pathophysiological contexts, especially as they relate to pancreas, very little is known about the regulation of the Reg1 gene expression. Miscellaneous factors including IL-6 and dexamethasone (13), PDGF and growth hormone (19), and gastrin (3) reportedly influence Reg1 levels in different cell types, partly through pertinent response elements on the gene promoter. Beyond autocrine or paracrine signals, regulation of protein production or RNA stability can be mediated by microRNAs (miRNA), which are short (18-25 nucleotides) single-stranded RNA molecules that cleave or translationally repress their specific target gene mRNAs (7). MicroRNA-modulated gene expression underlies diverse cellular processes including proliferation, apoptosis, differentiation and tumorigenesis. To this end, several microRNAs regulate pancreatic cell function, metabolism and differentiation. However, no miRNA has been identified to date that targets Reg genes in any cell type including pancreatic cells.

Here, we performed bioinformatic analysis of the 3' untranslated region (3'UTR) of the Reg1 mRNA seeking miRNAs with matching seed regions. The *mmu-miR-7a-5p* and *mmu-miR-7b-5p* featuring identical

seed regions were among the miRNAs identified as potentially targeting the murine *Reg1* mRNA. The aforementioned miRNAs belong to a group comprising *Mir7a-1*, *Mir7a-2* and *Mir7b* (9) all of which have the same seed sequence. The miR-7 family is evolutionary conserved across primates, rodents, and zebrafish exhibiting a neuroendocrine pattern of expression (4, 6, 9, 28, 47). In the adult rat and human pancreases, the islet/acinar tissue ratio of miR-7 expression is greater than 200 (6, 9, 26). Transgenic mice overexpressing miR-7a in β-cells develop diabetes due to impaired insulin secretion and β-cell dedifferentiation without significant changes in proliferation and apoptosis (29). In humans, miR-7 is also downregulated in PDAC and ampullary adenocarcinoma compared to normal pancreas (41).

Given the apparent inverse localization of miR-7 and Reg1 in the pancreatic acinar and islet compartments, we considered the possibility of miR-7 targeting Reg1 thereby influencing its expression in line with our *in silico* analysis. Here, we show that miR-7 is a negative regulator of the murine *Reg1*. The repression of Reg1 by miR-7 was observed in acinar cells and β-cells. Further, the human *REG1A* (homolog of the murine Reg1) and *REG1B* are not direct targets of miR-7, pointing to differences among species in the determinants of Reg expression. This is the first account of miRNA modulation of the expression of a member of the Reg protein family.

Materials and Methods

Housing and care of mice and collection of pancreatic tissues were approved by the Institutional Animal Care and Use Committee at Tufts University.

Murine pancreatic islet and exocrine tissue isolation

Isolation of pancreatic tissue was performed as we reported (50). Eight-week old C57/BL6 mice were injected intraperitoneally with 270 mg/kg ketamine/15 mg/kg xylazine (anesthesia overdose). A cannula was prepared by filling with collagenase solution (CIzyme, Vitacyte, Indianapolis, IN) a syringe fitted with a 27 G

needle. After an incision made in the lower abdomen (V cut), the pancreas was exposed, the pancreatic duct was clamped off at its duodenal insertion with a small bulldog clamp and the cannula was inserted into the duct proximal to the liver. Collagenase solution (3 ml/mouse) was injected to fully inflate the pancreas, which was subsequently removed and placed in a 50-ml conical tube for 20-30 min in a 37^{o}C water bath. At the end of the incubation, RPMI 1640 medium with 10% FBS was added (~20 ml) to each tube. The tubes were hand shaken vigorously for 5-10 seconds to break up the tissue and were kept in ice. Samples were washed three times to remove the collagenase by centrifugation at 180xg for 1.5 min. The supernatant was poured off, medium was added (~25 ml) and the samples were vortexed gently. The suspension was filtered through a 400-μm wire mesh (VWR, Randor, PA). The filtrate containing islets and exocrine tissue was spun at 180xg for 1.5 min and the supernatant was removed. The pellet was resuspended in 10-15 ml Histopaque 1077 (Sigma-Aldrich, St. Louis, MO) and vortexed until the suspension was homogeneous. After overlaying with 10 ml of RPMI 1640 medium the sample was spun for 20 min at 1750xg with very slow acceleration and no braking at 10^{o}C. Islets were collected from the interface with a 10-ml serological pipette and placed in a 50-ml conical tube. Exocrine tissue was collected from the bottom of the gradient and harvested to 50 ml-conical tubes. After three washes with RPMI 1640 with 10% FBS and centrifugation at 180xg for 1.5 min the islet fraction was transferred to 6-cm sterile culture dishes for islet picking under a microscope (Leica Microsystems Inc., Buffalo Grove, IL). Islet and the exocrine tissue were immediately processed for total RNA extraction or immunohistochemistry (see pertinent section below).

Cloning and Vector Preparation

The murine miR-7 (*mmu-miR-7a-2*) and 3'UTR of *Reg1* were cloned by high fidelity PCR (hfPCR) from genomic DNA isolated from MIN6 cells. The following primers were utilized: *mmu-miR-7a-2*; forward (F): 5'-ATATAGATCTGAAGGTGGCTAGCGTGA-3', reverse (R): 5'-CCCGTCGACTGAAATGACCAGCAC-3' (underlined: *BglII* and *SalI* sites). The miR-7 inhibitor (miR-7 sponge) sequence was designed (14) with two repeats of the miR-7 binding site: 5'-ACAACAAAATCACTAGTCTTCCA-3' and generated by hfPCR using

overlapping primers. The microRNA-67 from *C. elegans* (*cel-miR67*; accession MI0000038) was also included in the study as an unrelated microRNA control. The corresponding synthesized DNA (Eurofins Genomics, Louisville, KY) was used as a template for hfPCR amplification with the primers: (F) 5'-GGAGATCTATTCCAACTCGATCA-3', R: 5'-CCGTCGACAATAAACGAAATT-3' (underlined: *BglII* and *SalI* sites). The amplification product of miR-7, miR-7 inhibitor, or cel-miR-67 was ligated into the pSuper.GFP/neo vector (Oligoengine, Seattle, WA) between the *BglII* and *SalI* sites (pSuper.GFP/neo-miR-7).

The murine *Reg1* 3'UTR was cloned from MIN6 cell genomic DNA using the primers: F: 5'-GGGAATTCAGTCACCTGAAAAAAAATAGTCA-3', R: 5'-CCACTAGTGCAACATTGTAAAGGTGT-3' (underlined: *EcoRI* and *SpeI* sites). Genomic DNA was also extracted from human embryonic kidney 293 (HEK293) cells for cloning the 3'UTRs of *REG1A* and *REG1B* with the primers: *REG1A* 3'UTR; F: 5'-GGGAATTCAGGCAACTGGAAAATACATG-3', R: 5'-CCACTAGTGACAGCACAATAGTGGAAAC-3', and *REG1B* 3'UTR; F: 5'-GGGAATTCAGGAAGCTGAAAAATGGATGT-3, R: 5'-CCACTAGTGAGCAAATGCAGAAGACAGAA-3' (underlined: *EcoRI* and *SpeI* sites). The pGL3 luciferase vector (Promega, Madison, WI) was modified by (i) replacing the CMV promoter with the EF1α promoter and (ii) inserting a multiple cloning site (MCS) segment in the *XbaI* site flanking the 3' end of the luciferase gene. The MCS contained restriction sites for *EcoRI* and *SpeI* for insertion of the 3'UTR of *Reg1* (Luc-3'UTR-Reg1), *REG1A* or *REG1B*. A mutated *Reg1* 3'UTR sequence (^{97}-GTCTTCC^{-103} → ^{97}-GTCCCTT^{-103}; +1 denoting the nucleotide immediately after the *Reg1* stop codon) was generated by high-fidelity PCR using a corresponding synthesized template (Integrated DNA Technologies, Coralville, IA) and was inserted in the above luciferase vector (Luc-Δ3'UTR-Reg1) as described for the wild-type *Reg1* 3'UTR. All constructs were verified by sequencing.

Cell culture and transfection

MIN6 and βTC β-cells, 266-6 acinar cells (ATCC, Manassas, VA) and HEK293 cells were cultured in DMEM medium (Thermo Fisher Scientific) with 10% fetal bovine serum (FBS) and penicillin-streptomycin

(100 U/ml – 50 μg/ml). Cells were manually passaged every 5-7 days with TrypLE (Life Technologies, Carlsbad, CA) at a 1:4-1:6 ratio, and media were replaced every 3-4 days. The cultures were maintained in 5% CO_2/95% air at 37 ^oC. Cells were stained with Trypan Blue dye (Life Technologies) and counted in a hemocytometer or with the TC20 automated cell counter (Bio-Rad, Hercules, CA).

For transfection, cells were grown to 70-90% confluence and plasmids were delivered using Lipofectamine 2000 (Invitrogen). Transfection of miR7 (C-310592-07) or cel-miR67 (CN-001000-01) mimic (Dharmacon Inc., Lafayette, CO) was carried out with RNAiMax (Invitrogen, Carlsbad, CA) following the manufacturer's instructions.

RNA extraction, RT-PCR and quantitative PCR analysis

Total cellular RNA was extracted with Trizol (Life Technologies) according to manufacturer's instructions. Reverse transcription was performed at 42 ºC for 60 min with 1 μg total RNA using ImProm-II reverse transcriptase (Promega, Madison, WI) and 250 ng oligo$(dT)_{12-18}$ primers (Thermo Fisher Scientific). The reverse transcriptase was heat-inactivated at 70 °C for 15 min. The resulting complimentary DNA (cDNA) was analyzed on a StepOne Plus thermocycler (Applied Biosystems, Foster City, CA) by quantitative PCR (qPCR) for 40 cycles and 58-60 ºC annealing temperature depending on each primer set. The sequences for primers used in this study are listed in **Table 1**.

For miRNA expression analysis, total RNA was converted to cDNA with the qScript microRNA cDNA synthesis kit (Quantabio, Beverly, MA) using an oligo-dT adapter primer (**Table 1**). The resulting cDNA was amplified by qPCR (PerfeCTa SYBR Green SuperMix, Quantabio) with a universal PCR primer combined with a primer targeting the *mmu-mir7a-5p* or the *RNU6-2*.

Analysis was performed based on the $\Delta\Delta C_T$ method (17) with *Actb* and *RNU6-2* used as endogenous controls for the expression of *Reg1* and *mmu-mir7a-5p*, respectively.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 20 min and permeabilized with 0.1% Triton X-100 in PBS for 20 min at room temperature (nuclear marker staining). After three 5-min washes with PBS after each step, samples were blocked with 3% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in PBS for 30 min. Incubation was carried out at 4 ºC with a sheep anti-murine Reg1 antibody (AF1657; R&D Systems, Minneapolis, MN) followed by treatment with a donkey anti-sheep Rhodamine Red-X-conjugated antibody (713-295-147; Jackson ImmunoResearch) at room temperature for 1 h. Nuclear DNA was stained with DAPI (Sigma-Aldrich). Controls were stained with IgG instead of primary antibody. Immunostaining was visualized with a Leica TCS SPE confocal microscope (Leica Microsystems Inc., Buffalo Grove, IL).

Immunohistochemistry

Paraffin-embedded sections were dehydrated with increasing concentrations of ethanol (80–100%). After three 15-min washings with PBS, samples were blocked for 20 min in 3% NDS at 37°C and then incubated overnight at 4°C with guinea pig anti-murine insulin (A0564; Dako/Agilent Technologies, Carpinteria, CA) and sheep anti-murine Reg1 primary antibodies. Following three more washes with PBS, the sections were incubated with AffiniPure donkey anti-guinea pig Alexa Fluor 488 and anti-sheep Rhodamine Red-X (706-545-148 and 713-295-147, respectively; Jackson ImmunoResearch) secondary antibodies for 30 min at 37°C. The samples were then washed three times with PBS, mounted with SlowFade Diamond Antifade medium containing DAPI (Invitrogen) and visualized by confocal microscopy.

Western blot analysis

Total protein was isolated using lysis buffer containing Tris-HCl (50 mM, pH 8), NaCl (150 mM), NP40 (1%), sodium dodecyl sulfate (SDS) (0.1%), sodium deoxycholate (1%), protease inhibitor cocktail including PMSF, and phosphatase inhibitors (1 mM sodium fluoride, 5 mM sodium pyrophosphate, 5 mM sodium orthovanadate; all from Sigma-Aldrich). Protein concentration was determined via the Bradford method (Pierce

Biotechnology, Rockford, IL). Samples were separated in 10% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes as described before (25). The membranes were blocked with 5% skim milk and incubated overnight at 4 ^{o}C with diluted primary antibodies against murine Reg1 (1:1,000) and β-actin (1:1,000; #4970, Cell Signaling Technology, Beverly, MA). Then, membranes were washed four times (10-min) with TBST and incubated for 1 h at room temperature with corresponding horseradish peroxidase (HRP)-linked secondary antibodies (1:10,000; 713-035-147, 711-035-152, Jackson ImmunoResearch). After further washing with TBST, enhanced chemiluminescence reagent (Pierce Biotechnology) was added and the membranes were visualized with a C-DiGit blot scanner (LI-COR Biotechnology, Lincoln, NE). Densitometry was performed using the Image Studio software (LI-COR) (42).

Dual Luciferase Assay

For determination of luciferase activity, cells in 24-well plates were transfected with a total of 500 ng of plasmids per well as stated. Luciferase activity was assessed using the Dual Luciferase Assay Kit (Promega, Madison, WI) 24-72 h post-transfection. Luminescence was detected in a microplate reader (Spectramax i3x, Molecular Devices, San Jose, CA) with firefly luciferase activity normalized to the respective *Renilla* luciferase signal for each sample.

Enzyme immunosorbent assay (ELISA)

ELISA was performed to detect protein secreted in the culture medium. Cells were grown to 70-90% confluence and the medium was replaced by low-serum medium (DMEM with 1% FBS). After 30 h, the medium was collected and cells were harvested with TrypLE (Life Technologies, Carlsbad, CA) and resuspended in fresh medium. Cells were counted using a hemocytometer while the collected medium was added to 96-well EIA/RIA plates (Corning Inc, Corning, NY). Fresh low-serum medium served as control. Recombinant murine Reg1 (R&D Systems) was plated at different dilutions to obtain a standard curve as we reported (25). After overnight incubation at 4°C wells were coated with a blocking buffer of 3% BSA and 0.1%

azide in 0.1% TBST for 90 min at 37°C. Following four washes with 0.1% TBST, samples were incubated for 2 h at room temperature with biotinylated sheep anti-mouse Reg1 (1:2000; BAF1657, R&D Systems) diluted in buffer of 0.1% TBST with 1% BSA and 0.1% azide. Wells were again washed four times and a secondary rabbit anti-biotin antibody (#5597, Cell Signaling Technology) in the same dilution buffer was added for 1 h at room temperature. The wells were washed 4 times with 0.1% TBST and incubated with 1-Step™ Ultra TMB-ELISA substrate solution (Thermo Fisher Scientific) per manufacturer's instructions. Total Reg concentration was normalized by the number of cells in each sample.

Statistical analysis

Data are expressed as mean ± standard deviation (SD) unless stated otherwise from at least three independent experiments analyzed in triplicates. ANOVA and the *posthoc* Tukey test were performed using Prism (v. 8, GraphPad Software, La Jolla, CA). Values of $p<0.05$ were considered as significant.

Results

Murine Reg1 is a predicted target of miR-7

We set out to search *in silico* for miRNAs with binding sites on the 3'UTR of the murine *Reg1*. Interrogation of the TargetScan database (1) revealed several putative miRNAs targeting *Reg1* (**Table 2**). Of those miRNAs, the seed region of miR-7a-5p and miR-7b-5p was an exact match for the 97-103 nucleotide segment of the 3'UTR of *Reg1* (**Fig. 1A**). Given the involvement of the miR-7 family in pancreatic cell function and pathologies (15, 29), we decided to further investigate its potential role on the expression of murine Reg1.

Of note, we found no putative binding site(s) for miR-7 in the 3'UTR sequences of the genes encoding the human REG1A (Reg1 ortholog) and REG1B proteins despite the miR-7 family being conserved in mice and humans and the high homology between the murine and human Reg proteins.

Reciprocal expression of Reg1 and miR-7 in murine pancreatic exocrine and islet cells

We proceeded to investigate experimentally whether *Reg1* is a target of *miR-7*. First, the expression profiles of *Reg1* and *miR-7* were established by examining both exocrine cells and islet β-cells. Higher levels of *miR-7* were detected in MIN6 (7.08-fold, p=0.0164) and βTC β-cells (44.46-fold, p=0.009) than in 266-6 acinar cells (**Fig. 1B**). This miR-7 expression pattern was mirrored in murine primary islets (30.08-fold, $p=5x10^{-4}$) and exocrine tissue.

Conversely, *Reg1* expression was lower in β-cells than in 266-6 cells (MIN6: 26.9-fold lower, βTC: 12.4-fold lower, both with $p<10^{-4}$). Similarly, primary islets exhibited lower *Reg1* levels (4.97-fold, $p=7x10^{-4}$) compared to exocrine explants (**Fig. 1C**). Moreover, 266-6 cells were positive for Reg1 unlike MIN6 cells (**Fig. 2A**) and Reg1 was detected in exocrine regions but not in islets of pancreatic tissue sections (**Fig. 2B**). These findings were corroborated by Western blotting (**Fig. 2C**). Secreted Reg1 protein was present in supernatants from 266-6 cell cultures but was not detectable in those from MIN6 and βTC cells (**Fig. 2D**).

Taken together, these findings demonstrate that increased miR-7 expression coincides with lower levels of Reg1 in β-cells and primary islets while these profiles are inverted in exocrine cells.

Direct targeting of murine Reg1 by miR-7

The reciprocal expression of *Reg1* and *miR-7* in exocrine and endocrine cells along with the discovery *in silico* of a region of the *Reg1* 3'UTR matching the miR-7 seed sequence prompted us to investigate whether miR-7 represses *Reg1* directly. To this end, miR-7 was overexpressed or suppressed so that Reg1 levels could be analyzed. Overexpression of miR-7 (transfection with *miR7 OE* plasmid) led to a 60% reduction of *Reg1* expression (**Fig. 3A**; $p=5.5x10^{-3}$, n=3) in 266-6 cells. Accordingly, the amounts of Reg1 released in the medium were greater in control cells than in those overexpressing miR-7 (**Fig. 3B**). The content of Reg1 in the latter cells (miR7 OE) was almost half (**Figs. 3C-D**) of that of cells transfected with *cel-miR67*. The cel-miR-67 was chosen because (i) it is natively expressed in *C. elegans* reducing the risk for confounding interactions with murine cell miRNA moieties, and (ii) there are no apparent sites on the 3'UTR of Reg1 matching the cel-miR-67 seed region. The expression of cel-miR-67 did not affect Reg1 or miR-7 levels. Upon inhibition of miR-7 in

MIN6 cells, which natively exhibit pronounced *miR-7* levels, *Reg1* expression was increased (**Fig. 3A**) but there was no detectable change in Reg1 protein (**Fig. 3E**). Similarly, overexpression of miR-7 resulted in reduction of *Reg1* mRNA. These results show that the constructs used in this study were effective for miR-7 modulation. More importantly, augmenting or suppressing miR-7 levels causes significant changes in Reg1.

Direct repression of the expression of a gene by a particular microRNA involves binding of the latter to the 3'UTR of the target mRNA. Therefore, the 3'UTR of *Reg1* was placed downstream of the luciferase gene (Luc-3'UTR-Reg1). When Luc-3'UTR-Reg1 was delivered with a miR-7 mimic to 266-6 cells, luciferase activity was lower supporting the notion that miR-7 directly interacts with the 3'UTR of *Reg1* (**Fig. 4A**). No differences were noted in luciferase activity between cells treated with *cel-miR67* mimic or vehicle. Furthermore, 266-6 cells were co-transfected with the miR-7 overexpression plasmid and either the Luc-3'UTR-Reg1 vector or a luciferase construct with the *Reg1* 3'UTR but with its miR-7 binding site mutated (Luc-Δ3'UTR-Reg1). Despite overexpression of miR-7, luciferase signal was significantly reduced only in Luc-3'UTR-Reg1-transfected cells (**Fig. 4B**). Again, co-expression of *cel-miR67* with either of the aforementioned luciferase constructs did not result in differences in luciferase activity in comparison to cells receiving the empty expression vector.

These results prove that miR-7 directly targets the 3'UTR of murine *Reg1* in line with the bioinformatic analysis-based identification of *Reg1* as a miR-7 target.

Human REG1A and REG1B are not targets of miR-7

We also assessed whether the human REG1A (ortholog of murine Reg1) and REG1B, which together comprise the human REG1 subgroup, are also targeted by miR-7. Cells were transfected with a construct containing the luciferase gene upstream of the 3'UTR of the human *REG1A* (Luc-3'UTR-REG1A) or *REG1B* (Luc-3'UTR-REG1B). Additionally, these cells were co-transfected either with the miR-7 OE or empty (control) vector. There was no difference in luciferase activity in 266-6 cells receiving each luciferase vector regardless of the expression of miR-7 (**Fig. 5A**).

The findings support our *in silico* analysis indicating the absence of regions matching the miR-7 seed on the *REG1A* and *REG1B* 3'UTRs.

Discussion

Regulation of the expression of Reg family members remains puzzling despite their discovery almost four decades ago. This is surprising considering that Reg proteins are implicated in major pathologies of the pancreas and other tissues including the gastrointestinal tract and central nervous system. Here, we showed that miR-7 directly targets the murine *Reg1* modulating its expression. This is aligned with the reciprocal expression of miR-7 and Reg1 in the acinar and islet cells although the physiological significance of this pattern is unclear.

Both miR-7 and Reg1 influence the differentiation and proliferation of β-cells. Overexpression of *Mir-7a* in murine islets causes a decline in β-cell differentiation markers such as *Pdx1*, *Nkx6-1*, *Mafa* and *Neurod1* whereas pancreatic islets of *Rip-Cre Mir7a2*$^{fl/fl}$ mice exhibit higher steady-state levels of these transcripts compared to controls (29). MiR-7 expression drops during islet compensation at the onset of diabetes in BKS *db/db* mice (29). MiR-7 also regulates adult β-cell proliferation negatively as it targets five components of the mTOR signaling pathway (46). In contrast, Reg1 is considered a β-cell mitogen with its expression increasing in regenerating or hyperplastic islets although the exact mechanism is unknown (24, 45). Delayed development of diabetes and larger islet volume are observed in the offspring of NOD mice crossed with transgenic mice overexpressing *Reg1* from an insulin promoter (*Ins-Reg*) (44). After damage due to encephalomyocarditis virus infection, the islets of *Reg1*$^{-/-}$ mice exhibit lower BrdU labeling compared to those of wild-type mice (2). Moreover, diabetic NOD mice exposed to human REG1A display a greater β-cell mass than control animals (20). When combined with our findings of miR-7 targeting Reg1, a picture emerges of potentially coordinated Reg1/miR-7 activities. Further investigation will be necessary to determine if these activities have significant implications in the physiology for pancreatic cells. It will be particularly interesting to examine if control of murine β-cell proliferation by miR-7 may partly be realized through adjustment of Reg1 expression.

Expression of Reg1 has been detected in E9-E12 mouse embryos but not in E8.5 and Reg2, which exhibits a 76% amino acid homology with Reg1, appears on E12 (37). Conversely, miR-7 expression is low between E10.5-11.5 and increases at E13.5-E14.5 with strong localization in pancreatic endocrine cells and the neural tube (34). Of note, the *Reg2* 3'UTR exhibits no matching regions for the miR-7 seed based on bioinformatic analysis. This is in line with the restricted expression of Reg2 mRNA and protein in the exocrine tissue (40). Hence, the upregulation in Reg2 may compensate the repression of Reg1 due to rising miR-7 levels but the functional equivalence of Reg2 and Reg1 (despite their high amino acid homology) is not established.

It should be noted that the profile of Reg1 has not been studied in miR-7 mutant mice. As discussed already, deletion of *Mir7a2* in the islets of *Rip-Cre Mir7a2*$^{fl/fl}$ mice led to improved glucose tolerance via increased insulin secretion (29). However, the available gene expression data (GSE48195) are not sufficient for comprehensive statistical analysis to determine specific changes in *Reg1* levels. Moreover, mice with conditional excision of miR-7a-2 exhibit a reduction – but not complete ablation – of miR-7 expression most likely due to compensatory effects by other miR-7 family members (36). Hence, information about the relationship between Reg1 and miR-7 *in vivo* has been challenging to obtain from published studies involving miR-7 transgenic mice. This is further exacerbated by the plurality of miR-7 target genes.

Inhibition of miR-7 in β-cells resulted in increased *Reg1* expression but no changes were detected in the corresponding protein. A longer period of miR-7 suppression may be necessary for a substantial change in the protein level while additional mechanisms influencing the production of Reg protein cannot be ruled out.

The interaction between miR-7 and Reg1 may also be appreciated in other contexts beyond pancreas pathophysiology. MiR-7 is expressed in various regions of the brain of humans and mice (18, 28) and is linked to neuronal differentiation and function while its dysregulation may contribute to neurological disorders, including AD (38). To this end, aged animals have lower Reg1 expression than healthy animals in a murine model of AD (31). Hence, addressing whether miR-7 affects the expression of Reg1 in this context similar to what we have demonstrated in pancreatic cells is of significant interest. Similarly, Reg1 and miR-7 have been

implicated in different types of cancer in studies involving rodent models (23, 27, 35) opening prospects for further investigation of the Reg1/miR-7 relationship as a contributing factor.

We found that miR-7 directly targets the murine *reg1* but not the human *REG1A* and *REG1B*. Other genes also have miR-7 target sites in their 3'UTR that are poorly conserved among species. The human epidermal growth factor receptor (*EGFR*) gene features two segments targeted by miR-7 in its 3'UTR whereas the murine *Egfr* has a single 3'UTR region albeit with low similarity to the miR-7 seed (23). In fact, overexpression of miR-7 does not alter Egfr expression in MIN6 cells (46). The difference we report here between the murine Reg1 and the human REG1A and REG1B may underlie yet unknown disparities among species in the regulation of Reg and therefore in its function in normal and disease states. It also cautions about extrapolating findings on Reg proteins from studies involving rodents to humans. Nonetheless, the discovery of the modulation of a Reg family gene in murine cells by a miRNA motivates the exploration of a similar mechanism applicable to human Reg genes. To this end, ongoing work focuses on the identification of miRNAs targeting the human Reg members that may potentially lead to novel insights into Reg protein biology.

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Author Contributions

F. Zhang, S. Downing and Z. Chen performed research; F. Zhang, S. Downing, Z. Chen and E. Tzanakakis analyzed data; F. Zhang, S. Downing and E. Tzanakakis wrote the paper; E. Tzanakakis designed research.

Figure Legends

Figure 1. (A) Alignment of a putative miR-7 binding sequence in the murine *Reg1* 3'-UTR (nucleotides 97–103) is shown with the seed regions (capital letters) of mmu-miR-7a-5p and mmu-miR-7b-5p. Expression of (B) *mmu-miR-7* and (C) *Reg1* in MIN6 and βTC β-cells as well as primary islets and exocrine tissue. *$p<0.05$,

381 **p<0.01 vs. the expression of 266-6 cells (for MIN6 and βTC β-cells) or exocrine tissue (for islets) from n=4
382 separate experiments analyzed in triplicates. Normalization of *mmu-miR-7* and *Reg1* data was performed
383 relative to the expression of *RNU6-2* and *Actb*, respectively.
384
385 **Figure 2.** (A) Immunocytochemistry of 266-6 and MIN6 cells for Reg1. Nuclear DNA staining (DAPI) is also
386 shown along with merged images. Control samples (middle row) were stained only with the secondary
387 antibody. Bars: 25 μm. (B) Reg1 protein expression in pancreatic tissue. Insulin expression demarcates islet β-
388 cells. Bars: 50 μm. (C) Western blot for the expression of Reg1 in 266-6, MIN6, and βTC cells. The loading
389 control beta-actin (Actb) is also shown. L: ladder. (D) The concentration of Reg1 secreted in the culture
390 medium was determined by ELISA (n=3 experiments in triplicates), ND: not detectable.
391
392 **Figure 3.** (A) Relative *Reg1* expression in 266-6 and MIN6 cells transfected with either a *miR-7* overexpression
393 (OE) or inhibition (sponge; SP) plasmid. Results from cells transfected with a plasmid for an unrelated control
394 microRNA (*cel-miR67*) are included. *p<0.05, n=3 experiments vs. *Reg1* levels for cells transfected with empty
395 vector. The *miR-7* levels after transfection with *miR7* SP or *cel-miR67* plasmid are shown. * p<0.05, n=3
396 experiments vs. *miR7* expression for cells transfected with empty vector. (B) ELISA results showing the
397 amounts of Reg1 released by 266-6 cells transfected with either an empty vector (Control) or the miR-7 OE
398 plasmid. *p<0.05, n=3 experiments in triplicates. (C) A representative blot is shown of Reg1 content in 266-6
399 cells transfected with an *cel-miR7* or miR-7 OE plasmid. A MIN6 cell sample is also shown. Loading control:
400 Actb. (D) Results of densitometric analysis of western blots such as the one shown in (C). The results are the
401 average (n=3) values of the ratio of intensities of the Reg1 and corresponding Actb bands for each 266-6 cell
402 sample shown in (C). *p<0.05 vs. cells transfected with the *cel-miR67* plasmid. (E) Western blot for Reg1 from
403 266-6 and MIN6 cells transfected with either the empty miRNA expression vector (pSuper) or *miR-7* sponge
404 (*miR7* SP). The loading control, Actb, is also shown.
405

Figure 4. (A) Acinar 266-6 cells were transfected with a luciferase vector featuring the 3'UTR of Reg1 (Luc-3'UTR-Reg1) and incubated with various concentrations of miR-7 or cel-miR-67 mimic before measuring luciferase activity. Results are shown as relative activity compared to cells treated with vehicle only (0 nM). *p<0.0001 vs. cells transfected with cel-miR67 mimic at the same concentration, n=4-7 independent experiments in triplicates. (B) Luciferase activity was reduced with overexpression of miR-7 (miR7 OE, light bars) in cells co-transfected with Luc-3'UTR-Reg1 but not in those with the luciferase vector carrying the 3'UTR of Reg1 with a scrambled seed region (Luc-Δ3'UTR-Reg1). Dark bars depict results of cells transfected with an empty vector. *p<0.0001, n=3 experiments. No differences were noted in cells transfected with each of the aforementioned luciferase vectors and the cel-miR67 plasmid (hatched bars).

Figure 5. *miR7* does not target the 3'UTR of human REG1A and REG1B. Luciferase activity results are shown for 266-6 cells from n=3 independent experiments with triplicate measurements.

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Table 1. Primers used in this study (shown in a 5' to 3' orientation).

Gene	Forward primer	Reverse primer	Amplicon size (bp)
Reg1	CTCATGCCTGATCGTCCTGTC	AGCCCAAGTTAAACGGTCTTC	142
Actb	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC	245
Mir-7*	CGTGGAAGACTAGTGATTTTGTTG	miRNA reverse primer	86
Rnu6	GCAAATTCGTGAAGCGTTCC	miRNA reverse primer	104
miRNA reverse primer		GCATAGACCTGAATGGCGGTAAGGGT GTGGTAGGCGAGACATTTTTTTTTTTT TTTTTTTT	

* The primer is homologous to both Mir7-1 and Mir7-2.

Table 2. List of putative miRNAs targeting (mRNA) *reg1* based on *in silico* analysis.

miRNA	Sequence (3' to 5')	Targeted Positions of *reg1* 3'UTR
miR-19a/b-3p	AGUCAAAACGUACCUAAACGUGU	164-171
miR-7a/b-5p	UGUUGUUUUAGUGAUCAGAAGGU	97-103
miR-599-5p	AGAGGAUACAGUAGAAUAGUUU	75-81
miR-665-3p	UCCCUGGAGUCGGAGGACCA	101-107
miR-1197-3p	UCUUCAUCUGGUACACAGGAU	126-132
miR-463-3p	GAUGGAAUAUACCACAGAUAGU	73-79
miR-3101-5p	GAUCGAAAUCAGUUACCAUGG	132-138
miR-669o-5p	UGUAUUUGUACGUGUGUGUUGAU	168-174
miR-3097-5p	ACCUGUGUGUGAAGGGUGGACAC	137-143