MicroRNA-7 Regulates the mTOR Pathway and Proliferation in Adult Pancreatic β-Cells

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Elucidating the mechanism underlying the poor proliferative capacity of adult pancreatic β-cells is critical to regenerative therapeutic approaches for diabetes. Here, we show that the microRNA (miR)-7/7ab family member miR-7a is enriched in mouse adult pancreatic islets compared with miR-7b. Remarkably, miR-7a targets five components of the mTOR signaling pathway. Further, inhibition of miR-7a activates mTOR signaling and promotes adult β-cell replication in mouse primary islets, which can be reversed by the treatment with a well-known mTOR inhibitor, rapamycin. These data suggest that miR-7 acts as a brake on adult β-cell proliferation. Most importantly, this miR-7–mTOR proliferation axis is conserved in primary human β-cells, implicating miR-7 as a therapeutic target for diabetes. *Diabetes* 62:887–895, 2013

P ancreatic β-cell failure underlies the progression of all forms of diabetes (1). Thus, expansion of β-cells is a major goal of regenerative approaches to diabetes therapy, yet the extremely low replication rate of adult pancreatic β cells remains an enormous hurdle to overcome (2–6). Although signaling pathways that activate pancreatic β-cell proliferation have been extensively studied (7,8), an effective approach to increase adult β-cell replication still remains to be achieved. Thus, understanding the mechanism underlying the low capacity of adult β-cell replication under normal physiological conditions is crucial not only for a comprehensive view of adult β-cell growth but also for the development of new strategies for the treatment of diabetes.

MicroRNAs (miRs) are short, single-stranded RNA molecules that posttranscriptionally regulate gene expression by cleavage or translational repression of their specific target gene mRNAs. MicroRNA expression is often developmentally and tissue-specifically regulated and is involved in diverse biological processes, including development, cell proliferation, and metabolism (9–11). It has been shown that inhibition of microRNA maturation by conditional deletion of Dicer1 inhibits embryonic β-cell development and decreases insulin content in adult β-cells (12,13). In addition, individual microRNAs, including miR-375, miR-29, and miR124a, have specific functions in regulating insulin secretion, metabolism, and differentiation (14–16). However, no microRNA has been identified as a negative regulator associated with the low capacity of adult β-cell proliferation.

mTOR is an evolutionarily conserved serine/threonine protein kinase that plays a central role in cell growth and metabolism in response to a variety of environmental signals. mTOR exists in two distinct complexes, TORC1 and TORC2. Although TORC1 is involved in the regulation of numerous cellular processes related to growth and differentiation, TORC2 has a regulatory role in the cascade of insulin signaling. The functions of TORC1 and TORC2 are inhibited by rapamycin (17,18). Previous studies demonstrate that growth factors and nutrients stimulate pancreatic β-cell proliferation via the mTOR signaling pathway (19,20). The importance of mTOR signaling in regulating β-cell growth is further supported by genetic murine models. Activation of mTOR signaling by conditional activation of Rheb and Akt or deletion of TSC2 promotes pancreatic β-cell replication, expansion of β-cell mass, and improved glucose tolerance (21–23). In contrast, disruption of mTORC1 signaling in S6K1-deficient mice decreases β-cell mass and induces hyperglycemia (24).

miR-7 is one of the most abundant microRNAs in adult pancreas, with predominant expression in islet cells (25,26). Previous studies suggested miR-7 was involved in the modulation of cell growth (27–30). However, the role of miR-7 in adult β-cells is still unknown. Here, we show that miR-7/7ab family member microRNA-7a (miR-7a) is the major form of mature miR-7 expressed in adult pancreatic islets and that it targets multiple components of the mTOR signaling pathway in adult β-cells. Further, inhibition of miR-7 activates mTOR signaling and promotes adult β-cell proliferation in both mouse and human primary islets, suggesting that miR-7 functions as a negative regulator of adult β-cell proliferation and implicating miR-7 as a therapeutic target for the treatment of diabetes.

RESEARCH DESIGN AND METHODS

RNA isolation and real-time PCR. Total RNAs were prepared from cells with TRIzol reagent (Gibco BRL) and then reverse-transcribed with High Capacity *cDNA* Reverse Transcription Kit (Applied Biosystems). miRNA or Pri-miRNA assays were performed by TaqMan Real-Time PCR using the Taqman MicroRNA Reverse Transcription kit (Cat. No. 43667596; ABI) and specific Taqman MicroRNA Assays (assay IDs: miR-7a, 000268; miR-7b, 002555; snoRNA202, 001232; pri-miR-7a-2, Mm03307288_pri; and pri-miR-7a-1, Mm03307287_pri) according to the manufacturer's protocol. miRNA and Pri-miRNA levels were normalized by the levels of snoRNA 202 and hypoxanthine guanine phosphoribosyl transferase, respectively. Real-time PCR for mRNA was performed in triplicate using a Bio-Rad iCycler. Primers sequence information is as follows:

eIF4E-F: 5′-TGC TCA CGA TCT CAG CAT TC-3′
eIF4E-R: 5′-AAT GCA GCC CAA CAT AGA CC-3′
Mapkap1-F: 5′-TGT CTC CAA GTG GTG CTG AG-3′
Mapkap1-R: 5′-AGA GAG GCA GGC TGA CCA TA-3′
Mknk1-F: 5′-TTG CTC ACC GTG ATC TGA AG-3′
Mknk1-R: 5′-CAC TGC CCA AGT CAA AGT CA-3′
Mknk2-F: 5′-GGG ACA GTG CAC TTG ATT GA-3′
Mknk2-R: 5′-GGA GGG GTT TCT GAT TGT CA-3′
p70S6K-F: 5′-ACT GGA GCA CCT CCA TTC AC-3′
p70S6K-R: 5′-GTG TGA GGT AGG GAG GCA AA-3′

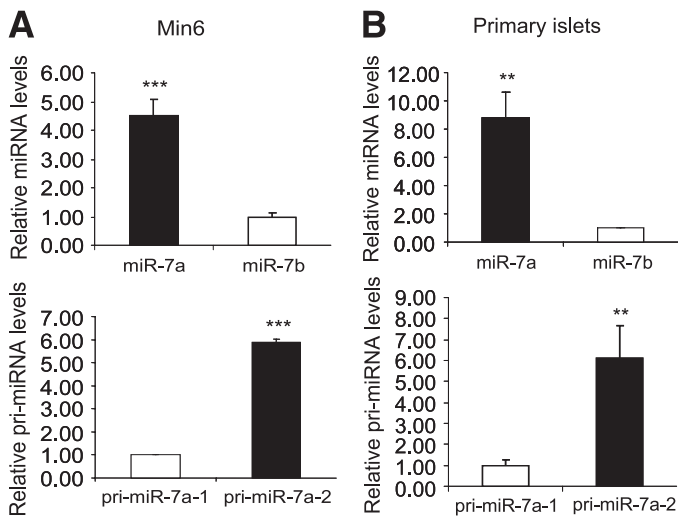**See accompanying commentary, p. 694.**

FIG. 1. miR-7a is the major miR-7/7ab microRNA family member in mouse pancreatic β-cells. Relative mRNA levels of mature miR-7 (7a and 7b; *upper panels*) and primary miR-7 (7a-1 and 7a-2; *lower panels*) in cultured Min6 cells (*A*) and wild-type islets isolated from 6- to 7-week-old C57/Bl6 mice (*B*), as determined by quantitative RT-PCR. $n = 3$, **$P < 0.01$, ***$P < 0.001$.

Construction of plasmids containing target gene 3′-untranslated regions. The 3′-untranslated regions (3′-UTRs) containing the conserved seed sequences of target genes (Fig. 2*B*) were amplified from Min6 cDNA with the following primers. The PCR products of target genes 3′-UTRs were cloned into *XhoI* and *NotI* sites of the luciferase reporter plasmid pMIR2-check2 (31). They are as follows:

P70s6k-UTR-F: 5′-ACT GCT CGA GTA AGC GGG GGA GAT AGA CCT-3′
P70s6k-UTR-R: 5′-ACT GGC GGC CGC TTG CAT AGG CAT GTG CTT TC-3′
eIF4e-UTR-F: 5′-ACT GCT CGA GGC AGA GTG GAC TGC ACT GAA-3′
eIF4e-UTR-R: 5′-ACT GGC GGC CGC AAT GCA GCC CAA CAT AGA CC-3′
Mknk1-UTR-F: 5′-ACT GCT CGA GCA CTG CAG GCA CTC CTT GTA-3′
Mknk1-UTR-R: 5′-ACT GGC GGC CGC ATT CCT CAT AGC AGG GCA CA-3′
Mknk2-UTR-F: 5′-ACT GCT CGA GCC CAC TTG GCT TTG TTT TGT-3′
Mknk2-UTR-R: 5′-ACT GGC GGC CGC AAG GAG TCC GGA GGT CAG TT-3′
MAPKAP1-UTR-F: 5′-ACT GCT CGA GTC CAC CAG CAT CCT GTA GTG-3′
MAPKAP1-UTR-R: 5′-ACT GGC GGC CGC TGT GAC AAG ATT TAA TGA CCA AA-3′

Pri-miR-7a-2 was amplified from Min6 cDNA with the following primers and cloned into the vector pCDNA3.1:

Pri-miR-7a-2-F: 5′-ATC GAA GCT TGG CAG GGT GCA ATG AGA ATA-3′
Pri-miR-7a-2-R: 5′-ATC GTC TAG ACC TTC TGA GGT TTC TCA ACT G-3′

Cell culture and transfection. Min6 and HEK-293T cells grown in 24-well plates were maintained with Dulbecco modified Eagle medium containing 10% FBS and 25 mmol/L glucose. HEK-293T cells were transfected with 450 ng of the PCDNA3.1 plasmid encoding pri-miR-7a-2 and 50 ng of the luciferase reporter construct containing the p70S6K, eIF4E, Mknk1, Mknk2, or Mapkap1 3′-UTRs and harvested 48 h later. Min6 cells were harvested 24 h after transfection with 50 nmol/L of antimiRNA-7a (miRCURY LNA microRNA inhibitor, has-miR-7 [411891–04]; Exiqon) or control oligonucleotide (miRCURY LNA microRNA inhibitor, Negative Control A [199004–04]; Exiqon) and 50 ng luciferase reporter. All transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega).
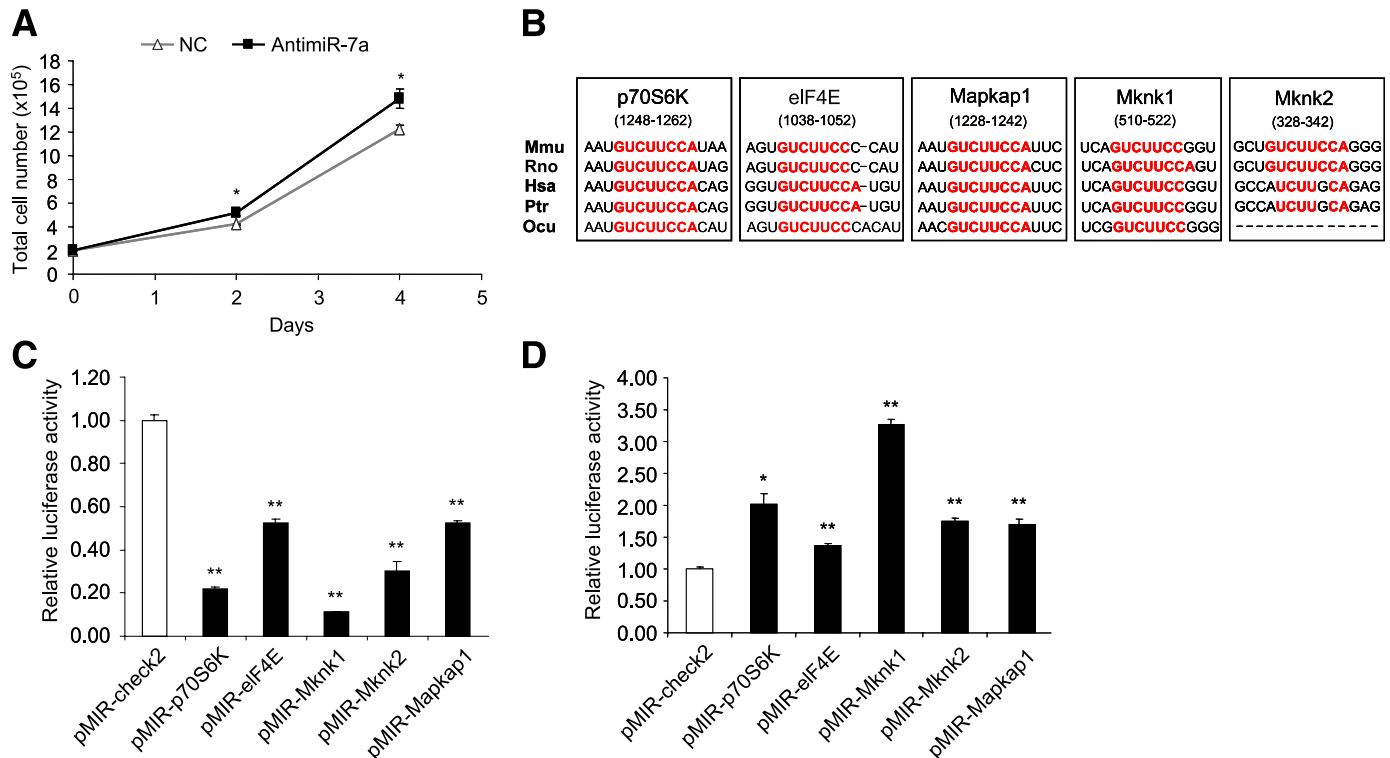

FIG. 2. miR-7a acts on the 3′-UTR of genes involved in the mTOR pathway. *A*: Growth curve analysis of miR-7a-deficient Min6 cells. Min6 β-cells were transfected with control (NC) or 7a oligonucleotide. Total cell number was counted on days 0, 2, and 4. $n = 3$, *$P < 0.05$. *B*: The predicted miR-7 binding site consensus in the 3′-UTR of genes involved in the mTOR pathway using TargetScan software and evolutionary conservation. Mmu, Mus musculus; Rno, Rattus norvegicus; Hsa, Homo sapiens; Ptr, Pan Troglodytes; Ocu, Oncorhynchus. *C*: Reporter assays performed in HEK-293T cells cotransfected with a plasmid encoding pri-miR-7a-2 and the luciferase reporter vector (pMIR-check2) containing the 3′-UTR for p70S6K, eIF4E, Mknk1, Mknk2, or Mapkap1 ($n = 3$, **$P < 0.01$). *D*: Reporter assays performed in Min6 cells cotransfected with antimiR-7a inhibitor and the same 3′-UTR luciferase reporter constructs ($n = 3$, *$P < 0.05$, **$P < 0.01$). (A high-quality color representation of this figure is available in the online issue.)

Primary mouse islet experiments. Mouse islets were isolated from 6- to 7-week-old wild-type C57/Bl6 males using collagenase digestion as previously described (32). Isolated islets were trypsinized (0.05% Trypsin) and plated as a monolayer onto poly-L-lysine-coated six-well dishes or four-well chambers, followed by transfection (Lipofectamine 2000) with 40 nmol/L antimiR-7a or control oligonucleotide. Islets were cultured in RPMI-1640 medium and harvested at 48 h after transfection.

Primary human islet experiments. Human islets were provided by the University of Pennsylvania Human Islet Isolation Laboratory, dispersed as described for mouse islets, and maintained in CMRL-1066 supplemented medium for 24 h, followed by transfection with 40 nmol/L antimiR-7a or control oligonucleotides by Lipofectamine 2000 (Invitrogen) and harvested 48 h later. Islets obtained from four normoglycemic human donors, two males ages 15 and 24 years and two females ages 48 and 49 years, were prepared and transfected as for mouse islets.

Western blot analysis. Total protein lysates were prepared by 1% NP40 lysis buffer and 4 μg total protein samples were separated by SDS-PAGE on 4–12% gels (Invitrogen). Immunoblotting was performed with the following primary antibodies, according to the manufacturer's protocol. Anti-p70S6K (1:5,000 dilution) was a gift from Dr. Morris Birnbaum. Other antisera were purchased commercially as follows: anti-Mapkap1 (05–1044, 1:1,000 dilution; Millipore), anti-

Mknk2 (S-20, 1:500 dilution; Santa Cruz Biotechnology), and anti-Pax6 (PRB-278P, 1:5,000 dilution; Covance); all other antisera were purchased from Cell Signaling (1:1,000 dilution). The results of at least three independent experiments were quantified using Image J software (National Institutes of Health).

Immunofluorescence. Transfected islet cells grown in chamber slides were fixed with 4% paraformaldehyde and costained with guinea pig anti-insulin (1:1,000 dilution; Linco) and rabbit antiphospho-Histone H3 (1:500 dilution; Upstate Biotech) or rabbit anti-Ki67 (1:1500 dilution; Leica). For the apoptosis assay, TUNEL staining was performed using the Apoptag Peroxidase in Situ Apoptosis Detection Kit (Chemicon) and according to manufacturer's instructions. Images were captured with iVision software (BioVision Technologies) using constant exposure parameters for each fluorescence channel.

Insulin secretion. Forty-eight hours after transfection, islet cells were incubated in Krebs-Ringer bicarbonate HEPES buffer for 30 min and then cultured in 2.8 mmol/L glucose and 16 mmol/L glucose for 30 min each. Secreted insulin and insulin content was analyzed by ELISA (Crystal Chem).

Statistical analysis. All data represent the mean ± SE. For all comparisons, including quantitative RT-PCR and quantification of β-cell proliferation, statistical significance was assessed using a two-tailed Student t test. Differences were considered significant if $P < 0.05$.
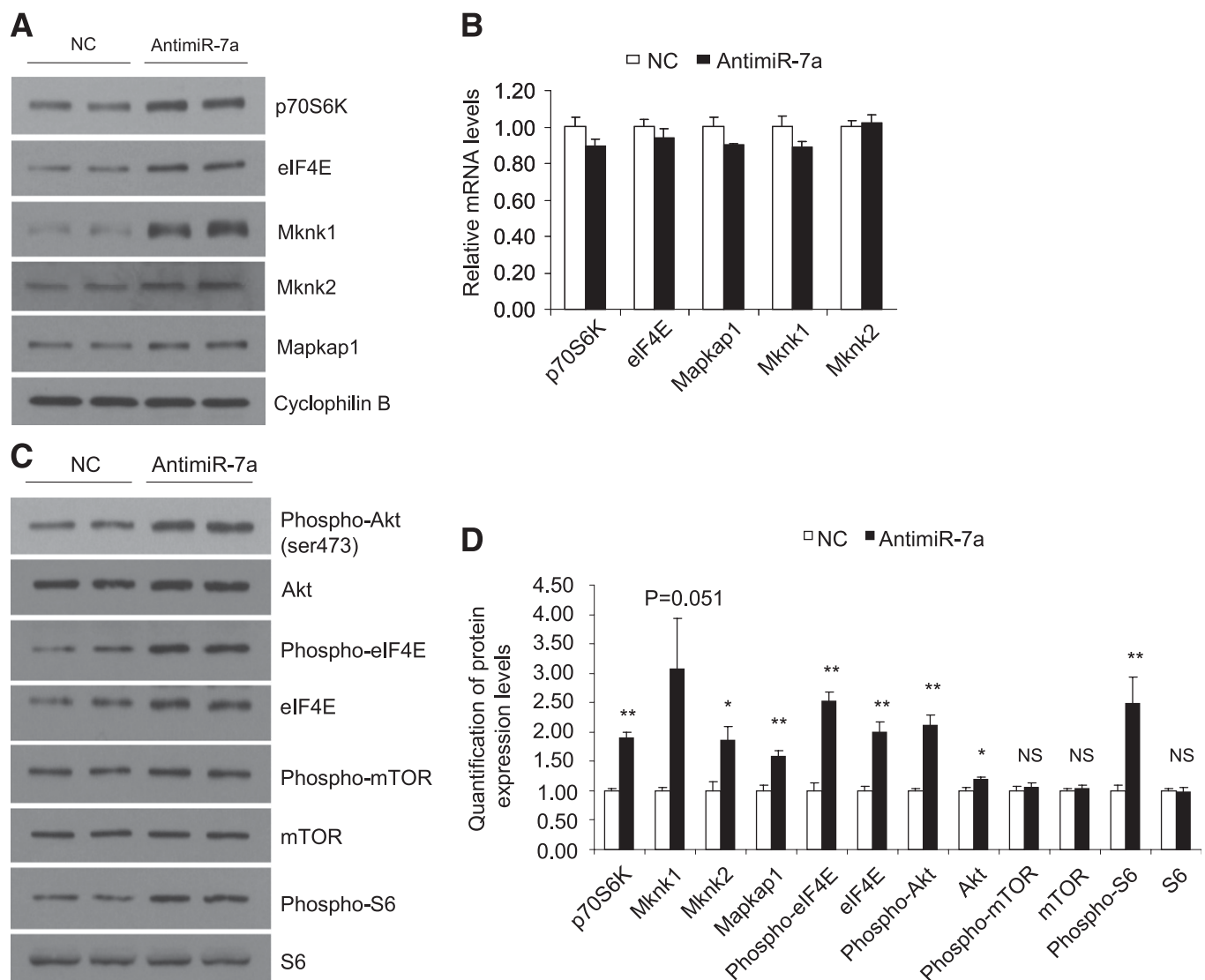

FIG. 3. Inhibition of miR-7a activates the mTOR signaling pathway in Min6 β-cells. *A* and *C:* Representative Western blot analysis of the indicated mTOR pathway proteins in Min6 cells transfected with control (NC) or antimiR-7a oligonucleotide, performed in duplicate. Cyclophilin B protein was detected as a loading control. The same loading control applies to (*A*) and (*C*) and is only shown once in (*A*). *B:* Quantitative RT-PCR analysis of relative gene expression levels of potential miR-7a targets in antimiR-7a-transfected Min6 cells; all the *P* values were not statistically significant relative to NC. *n* = 3. *D:* Quantification analysis of Western blot results shown in (*A*) and (*C*). *n* = 4, *P < 0.05, **P < 0.01. NS, no statistical significance.

RESULTS

miR-7a is the major miR-7/7ab microRNA family member in mouse pancreatic islets and acts on the 3′-UTR of genes involved in the mTOR pathway. miR-7 belongs to the highly evolutionarily conserved miR-7/7ab microRNA family and is one of the most abundant microRNAs in both rodent and human adult pancreas, with predominant expression in islet cells (25,26). Mouse mature miR-7 consists of miR-7a and miR-7b, which are encoded by three genes in the mouse genome, miR-7a-1, miR-7a-2, and miR-7b (33). In both Min6 β cells and primary mouse islets, miR-7a is the major form of mature miR-7 and miR-7a-2 is the major precursor expressed (Fig. 1*A*, *B*). To elucidate the role of miR-7a in pancreatic β-cells, we blocked miR-7a activity in Min6 cells using an antimiR-7a oligonucleotide inhibitor. A slight but significant increase of cell growth was observed in miR-7a-deficient Min6 cells compared with the control oligonucleotide (Fig. 2*A*), suggesting that miR-7a may negatively regulate cell proliferation. In contrast, growth curve analysis after overexpression of miR-7a revealed only a small nonsignificant reduction in cell growth, which could indicate that the high endogenous miR-7a levels in Min6 cells have exerted their maximal response (data not shown).

The mTOR signaling pathway functions as a central mediator of cell growth and metabolism. Remarkably, we found that five components of the mTOR signaling pathway are potential targets of miR-7a identified using TargetScan software (34), including the two main downstream effectors of TORC1, p70S6K and eukaryotic translation initiation factor 4E (eIF4E), two MAPK-interacting kinases, Mknk1

and Mknk2, that phosphorylate eIF4E, and one of the essential TORC2 components, Mapkap1. The miR-7a binding site consensus sequences found in the 3′-UTRs of these five genes are highly evolutionarily conserved (Fig. 2*B*). To demonstrate whether these 3′-UTRs are actual targets of miR-7a, HEK-293T cells were cotransfected with the miR-7a precursor miR-7a-2 and luciferase reporters containing the 3′-UTR of each predicted target. The miRNA expression assay confirmed the successful production of mature miR-7a from the overexpressed miR-7a-2 precursor (Supplementary Fig. 1). Induction of miR-7a significantly downregulated 3′-UTR reporter activity for all five targets (Fig. 2*C*), whereas inhibition of miR-7a by antimiR-7a in Min6 cells increased the activity of these same reporters (Fig. 2*D*). These results indicate that miR-7a acts on the 3′-UTR of multiple components of the mTOR signaling pathway and thereby may modulate the expression and function of these targets.

Inhibition of miR-7a activates mTOR signaling in Min6 β-cells. MicroRNAs are posttranscriptional regulators that complementarily bind to the RNA transcripts of target genes and affect gene expression through translational repression, mRNA destabilization, and degradation (35). Accordingly, Western blot analysis revealed increased protein levels of p70S6K, eIF4E, Mknk1, Mknk2, and Mapkap1 in Min6 cells transfected with antimiR-7a inhibitor (Fig. 3*A*, *D*). Quantitative RT-PCR analysis showed no statistical difference in expression at the mRNA level (Fig. 3*B*), suggesting primarily translational repression of these targets by miR-7a. Mapkap1 is an essential TORC2 subunit required for complex integrity and for Akt/PKB phosphorylation at
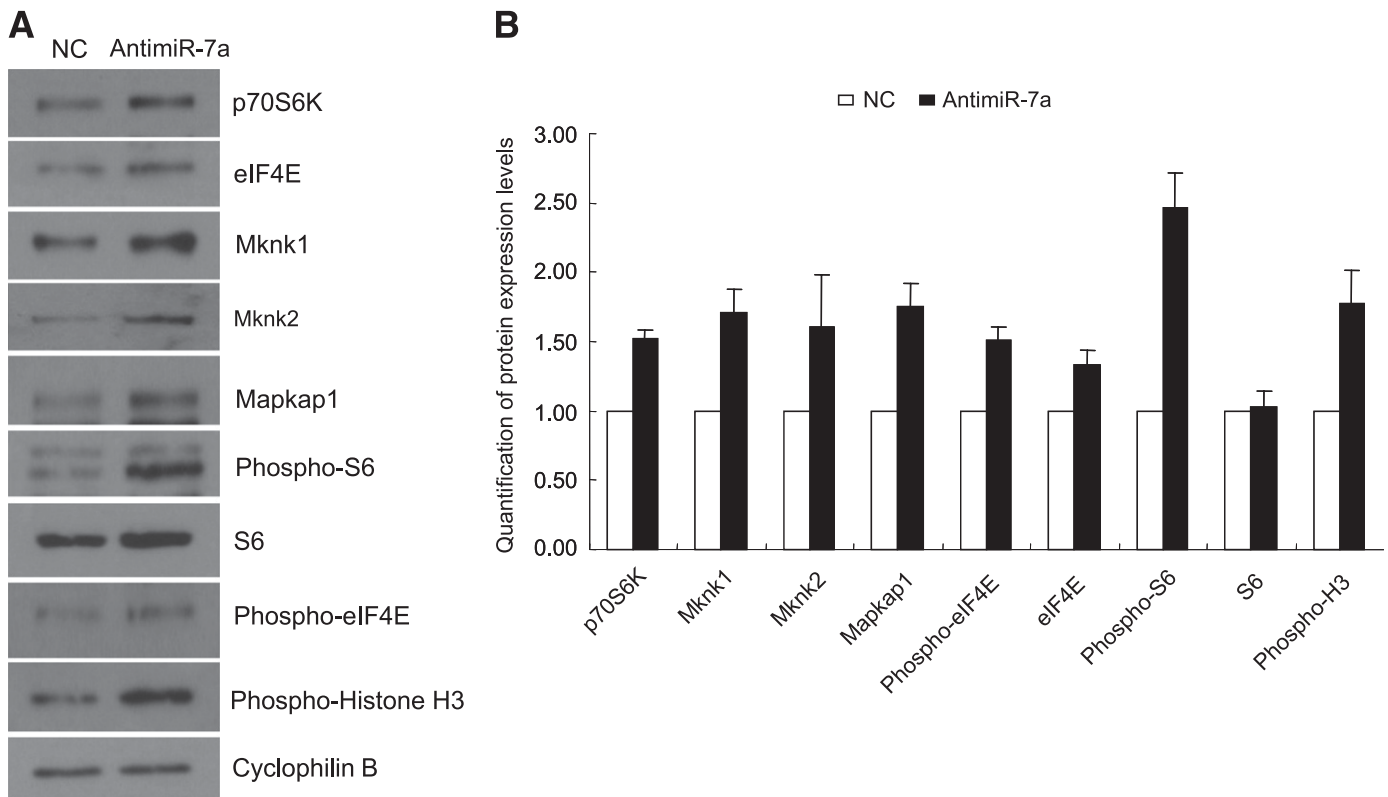

FIG. 4. miR-7a targets mTOR signaling in primary mouse islets. *A*: Representative Western blot analysis of lysates prepared from primary islets transfected with antimiR-7a or control oligonucleotide for the indicated miR-7 targets, phospho-S6 and phospho-eIF4E, as well as the proliferation marker phospho-Histone H3. *B*: Quantification analysis of Western blot results from three independent experiments is shown in (*A*). The value of each control group was set to 1.00.

Ser473 (36). Consistent with increased Mapkap1 protein levels, the phosphorylation of Akt/PKB at Ser473 was increased in miR-7a-deficient Min6 cells (Fig. 3C, D). Although the amount of total Akt is significantly increased, the increase is minor compared with the increase in Akt phosphorylation. Inhibition of miR-7a did not affect the phosphorylation of mTOR in Min6 cells, although a previous study suggested that activation of Akt/PKB increases mTOR phosphorylation by suppressing TSC2 (37). In contrast, the phosphorylation of S6, a direct downstream target of p70S6K, was clearly increased in antimiR-7a inhibitor transfected Min6 cells (Fig. 3C, D). We also observed an increase in eIF4E phosphorylation at Ser209 in miR-7a-deficient Min6 cells (Fig. 3C, D), consistent with increased total eIF4E and Mknks levels (Fig. 3A) and previous reports establishing eIF4E as an Mknk substrate (38,39). These results demonstrate that inhibition of miR-7a activates mTOR signaling in Min6 cells.

miR-7a deficiency activates the mTOR signaling pathway and promotes cell proliferation in mouse islets. Next, we determined whether miR-7a regulation of mTOR signaling is conserved in primary islets. Dispersed adult mouse islet cells were transfected with fluorescently labeled antimiR-7a inhibitor or control oligonucleotide, resulting in >90% transfection efficiency. Western blot analysis showed increases in the levels of p70S6K, eIF4E, Mknk1, Mknk2, and Mapkap1, as well as in the phosphorylation of S6 and eIF4E in miR-7a-deficient mouse islets (Fig. 4A, B) demonstrated that suppression of miR-7a activated mTOR signaling in primary islets. Phospho-Akt levels were not detectable in mouse islets because of the low yield of protein in our dispersed islet lysates. We also observed a dramatic increase in expression of the mitosis marker, phospho-histone H3, indicating that silencing of miR-7a promotes primary islet cell proliferation (Fig. 4A, B). Immunofluorescence analysis further showed that the number of insulin-positive cells expressing phospho-Histone H3 and Ki67 increased by 2.6-fold and 2.9-fold, respectively, in islets transfected with the antimiR-7a inhibitor (Fig. 5A–D), demonstrating that miR-7a negatively regulates adult β-cell proliferation.

Cells undergoing replication may be at increased vulnerability to undergo apoptosis (40); however, TUNEL staining revealed no change in the low rate of β-cell apoptosis in miR-7a-deficient primary islets (Fig. 5E). To determine whether miR-7a deficiency affects β-cell secretary function, we performed glucose-stimulated insulin secretion assays and found no difference in insulin secretion and content between miR-7a-deficient and control islet cells (Fig. 5F, G).

The miR-7–mTOR-proliferation axis is conserved in primary human β-cells. The miR-7 binding consensus sequences in the p70S6K, eIF4E, Mapkap1, and Mknk1 genes are highly conserved between mouse and human (Fig. 2B). Compared with mouse mature miR-7, which
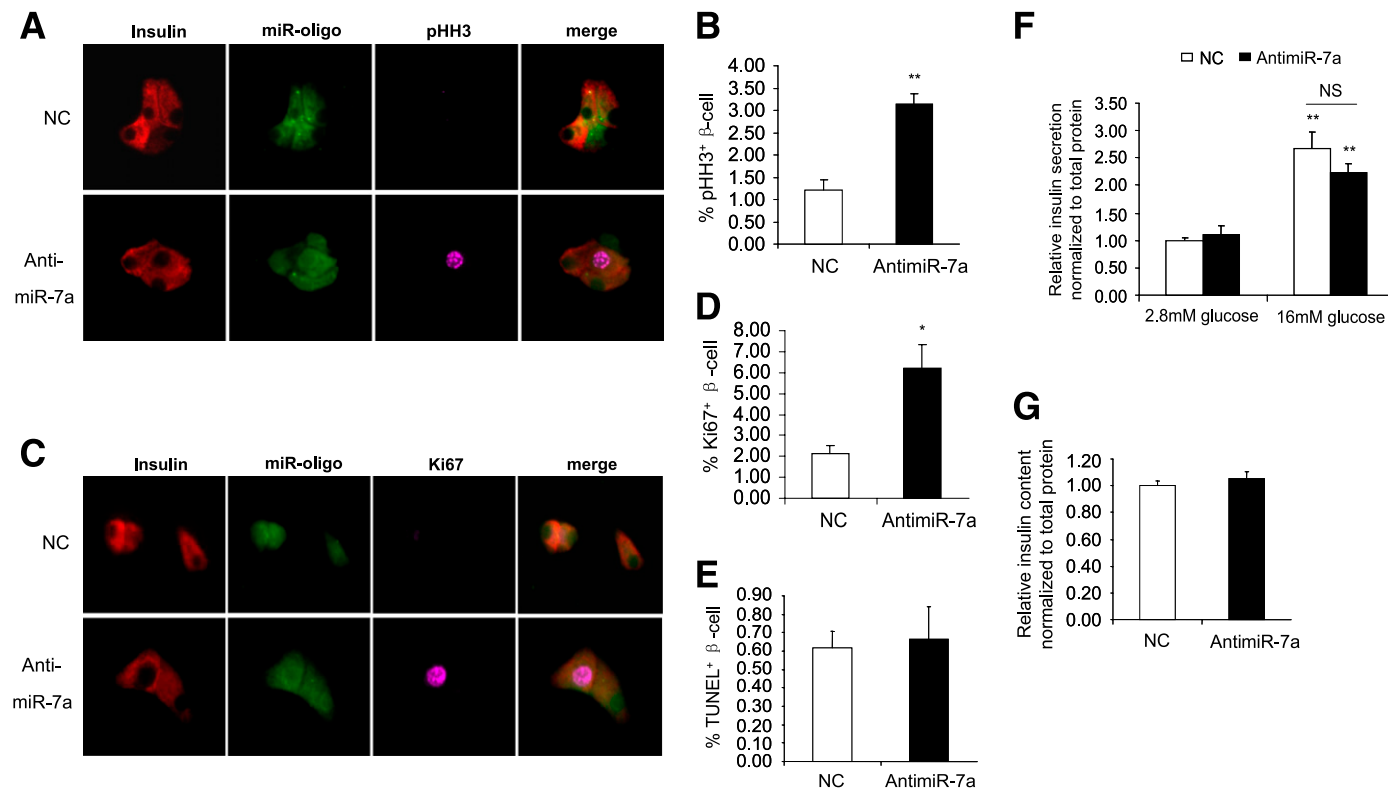

FIG. 5. miR-7a regulates cell proliferation in primary mouse islets. A–D: Representative images of primary mouse β-cells costained with anti-insulin (red) and antiphospho-Histone H3 (magenta) (A) or anti-Ki67 (magenta) (C). Fluorescently labeled control (NC) or antimiR-7a oligonucleotide are shown in green. Percent adherent-transfected insulin-positive phospho-Histone H3 (B) or Ki67-positive (D) cells were quantified in 500 insulin-expressing cells per group (n = 3, *P < 0.05, **P < 0.01 relative to NC). E: miR-7a deficiency did not induce β-cell apoptosis in mouse islets. miR-7a inhibitor (antimiR-7a) or control oligonucleotide (NC) transfected mouse islets cells were costained with TUNEL and insulin. Total numbers of TUNEL-positive cells were quantified in 500 insulin-positive cells per group; there is no significant change between the groups (n = 3). F and G: miR-7a deficiency did not alter glucose-stimulated insulin secretion (GSIS) in mouse islets cells. Relative insulin secretion at <2.8 mmol/L or 16 mmol/L glucose and insulin content were shown. n = 3, **P < 0.01 relative to 2.8 mmol/L. NS, no statistical significance.

exists in two homologous isoforms, there is only one mature isoform of miR-7 in humans, encoded by three different loci. Importantly, human miR-7 shares sequence identity with mouse miR-7a (33). To investigate the role of miR-7 in human β-cell proliferation, we transfected dispersed human islet cells with control oligonucleotide or antimiR-7a inhibitor. Only 0.01% of Ki67-positive β-cells were detected in the control group, in agreement with previous observations (41). In striking contrast, the number of Ki67-positive insulin-expressing human β-cells increased nearly 30-fold in the setting of miR-7a deficiency (Fig. 6A, B). As in mouse islets, miR-7 deficiency caused an activation of mTOR signaling in human islets. Both total and phosphorylated eIF4E protein levels were upregulated by suppression of miR-7, as was the level of phosphorylated S6 (Supplementary Fig. 2). These findings indicate that miR-7a negatively regulates the mTOR pathway and β-cell replication in human islets.

mTOR signaling is the key pathway mediating miR-7 modulation of primary β-cell proliferation. Recently, several other targets of miR-7 have been reported. The critical developmental transcription factor Pax6 is a newly identified target of miR7 during pancreas development (42). We found that Pax6 also is regulated by miR7 in Min6 cells (Supplementary Fig. 3A, B) and in adult islets (Supplementary Fig. 3D, E). Consistent with the regulatory pattern of identified targets in the mTOR pathway, miR-7a deficiency did not affect the mRNA levels of Pax6 (Supplementary Fig. 3C). Epidermal growth factor receptor (EGFR) also has been recognized as a target of miR-7 in the modulation of cell growth in human cancer cells (27,28); however, our bioinformatics analysis did not reveal

conservation of the target seed sequence in the 3′-UTR of murine EGFR. Accordingly, Western blot analysis demonstrated that EGFR protein levels were not affected by inhibition of miR-7a in Min6 cells (Supplementary Fig. 4), suggesting that the increased Min6 cell growth observed after miR-7 inhibition was not mediated by an increase in EGFR expression.

Given the likely possibility that miR7 regulates known and as yet unknown target mRNAs in β-cells, it became necessary to determine whether miR-7 modulation of mTOR signaling is required for its effect on β-cell replication. We treated dispersed islet cells with a specific inhibitor of mTOR, rapamycin, after the transfection with antimiR-7a inhibitor or control oligonucleotide. Quantification of Ki67-positive insulin-expressing cells showed that rapamycin abrogated the effect of miR-7a deficiency on β-cell replication in both mouse (Fig. 7A) and human islets (Fig. 7B), indicating that enhanced mTOR signaling is required for the effect of miR-7a inhibition on β-cell replication.

DISCUSSION

Although the signaling pathways that can activate β-cell proliferation have been extensively studied, the factors limiting replication of adult β-cells are less known. Here, we show that miR-7a, a pancreatic β-cell-enriched microRNA, inhibits multiple components of the mTOR signaling pathway (Fig. 7C). Silencing of miR-7 activated mTOR signaling and promoted pancreatic β-cell proliferation in an mTOR-dependent manner. To our knowledge, this is the first report that uncovers a negative
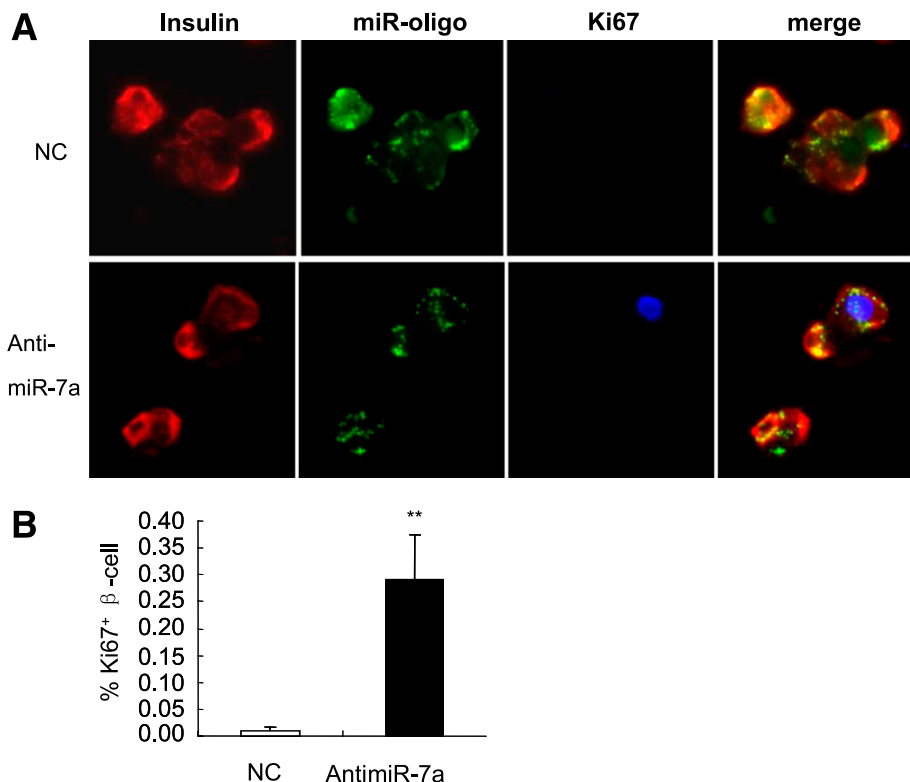

FIG. 6. miR-7 deficiency promotes replication of human islet β-cells. Primary islets isolated from normoglycemic donors (15- and 24-year-old men, 48- and 49-year-old women, respectively) were prepared and transfected as for mouse islets. A: Representative images of insulin-positive (red) and Ki67-positive (blue) cells transfected with control (NC) or antimiR-7a oligonucleotide (green). B: Percent Ki67-positive cells were quantified in 6,000 insulin-expressing cells per group ($n = 4$, **$P = 0.01$ relative to NC).
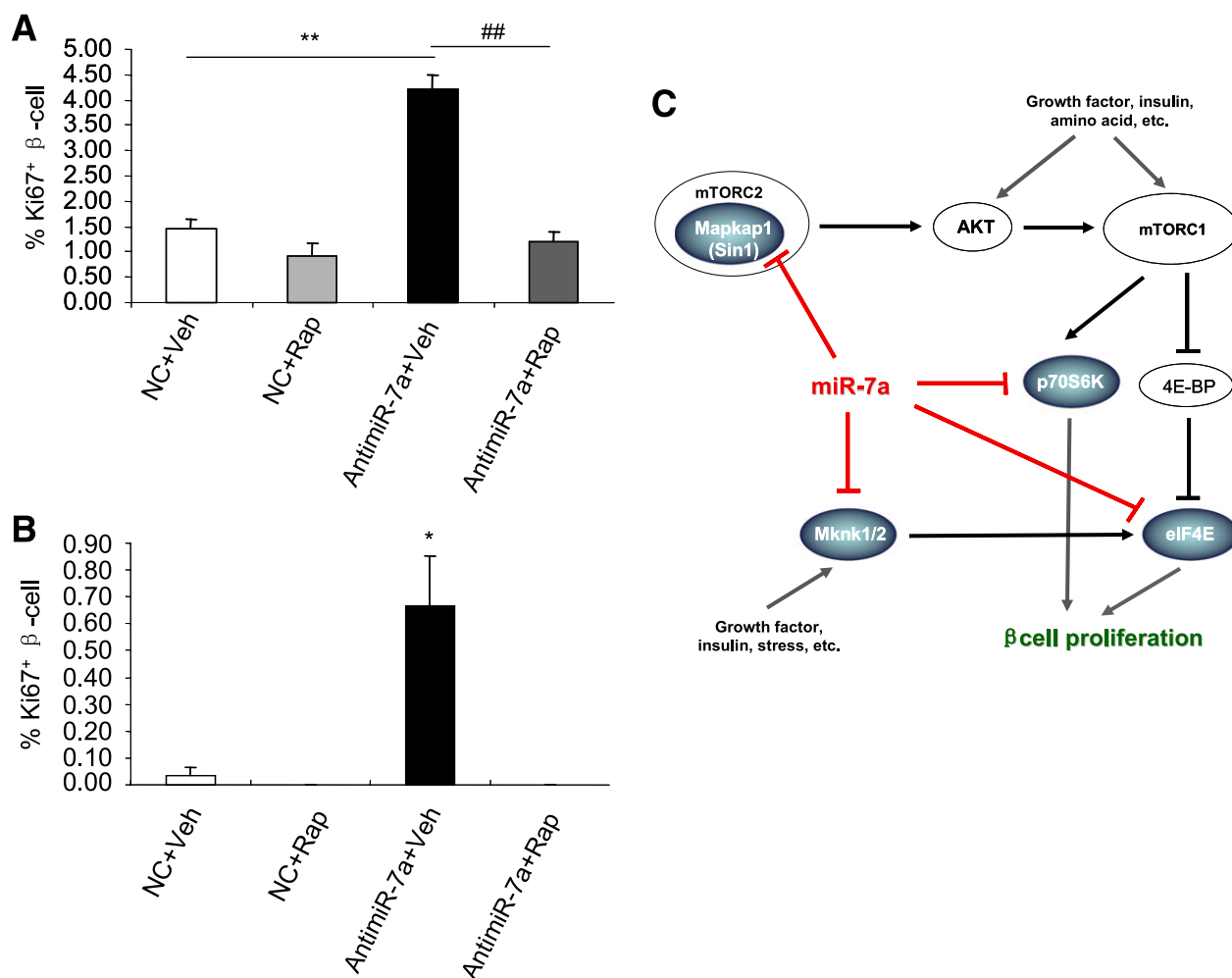
FIG. 7. The mTOR inhibitor, rapamycin, blocks β-cell proliferation induced by miR-7 deficiency in primary islets. After transfection with miR-7a inhibitor or control (NC) oligonucleotide as described, islet cells were treated with 10 nmol/L rapamycin for 24 h and fixed for costaining with Ki67 and insulin. *A*: Percent Ki67-positive cells were quantified in 1,000 insulin-expressing mouse islets cells per group ($n = 3$, **$P < 0.01$, ## $P < 0.01$). *B*: Primary islets were isolated from normoglycemic donors (a 48-year-old woman and 15- and 24-year-old men, respectively). Ki67-positive cells were quantified in 6,000 insulin-expressing cells per group ($n = 3$, *$P < 0.05$). *C*: Schematic model for miR-7a regulation of the mTOR signaling pathway. Inhibitory relationships are depicted in red, activating relationships are depicted in black/gray. Direct targets of miR-7a are shaded. (A high-quality color representation of this figure is available in the online issue.)

regulatory role for a microRNA in adult pancreatic β-cell proliferation.

Recent studies implicate other signaling pathways as modulated by miR-7 in other cell types (27–30). Although it is recognized that modulation of mTOR signaling affects pancreatic β-cell growth (19–23), it was important to directly test the role of mTOR signaling in the ability of miR-7 inhibition to promote β-cell replication. The outcome of rapamycin to abrogate the enhancement of β-cell replication establishes the central role of mTOR signaling in this response.

Based on a previous report that miR-7 can target human EGFR (28), and based on the knowledge that EGFR is upregulated in hepatocellular carcinoma cells, Fang et al. (43) used miR-7 overexpression to demonstrate targeting of PIK3CD in hepatocellular carcinoma cells. It was further suggested that mTOR and p70S6K transcripts also represent direct targets of miR-7; however, the relevance of miR-7 indirectly regulating mTOR and p70S6K is unclear, given that the observed transcriptional effects as well as regulation of Akt transcripts were fully recapitulated by

siRNA-mediated silencing of the primary target identified, PIK3CD. Importantly, we did not find regulation of total Akt or mTOR levels by miR-7a manipulation in β-cells (Fig. 3*C*, *D*), and even the p70S6K regulation that we observed in β-cells was clearly posttranscriptional, in contrast to the observations in hepatocellular carcinoma cells.

miR-7 is well-expressed during pancreas development (25,44), and inhibition of miR-7 during early pancreas development by intrauterine fetal heart injections of antisense morpholinos results in decreased β-cell numbers and glucose intolerance in the postnatal period (44). Our observation that miR-7 inhibition promotes β-cell replication suggests a distinct regulatory role for miR-7a in adult compared with developing β-cells. Although we cannot exclude that miR-7 targets genes during pancreas development distinct from those targeted in adult β-cells, activation of the mTOR signaling pathway by L-Leucine during pancreas development has remarkably similar effects as those reported with miR-7 inhibition during development, and the effects of L-Leucine are inhibited by rapamycin (45). These data suggest that miR-7, acting on

the mTOR signaling pathway, may play strikingly distinct roles in the developing pancreas as compared with the mature pancreas.

It is now appreciated that despite very low proliferation rates in primary β-cells, replication is the primary means of β-cell renewal and expansion in adult organisms, and even apparently small changes in this rate can have a major impact on the functional mass of β-cells over the course of the long term. Unlike rodent β-cells that can be induced to replicate using a variety of strategies, including partial pancreatectomy, induction of obesity and insulin resistance, infusion of glucose, administration of growth factors, and activation of signaling pathways downstream of these growth factors and nutrients, application of these strategies has not led to robust rates of proliferation in adult human β-cells (34,46–49). Only direct adenoviral manipulation of the cell-cycle machinery leads to BrdU incorporation into as much as 10–15% of insulin expressing human β-cells (46). Thus, even the low rate of human β-cell replication induced by miR-7 inhibition may have a clinically significant impact on β-cell numbers.

In conclusion, our finding that miR-7 inhibition promotes mTOR signaling and thereby induces β-cell replication in human islets provides a mechanistic explanation for the poor proliferative capacity of adult β-cells and implicates miR-7 as a novel target for diabetes therapy. We speculate that inhibition of miR-7 in vivo or ex vivo could be optimized and exploited to expand β-cell numbers to improve glucose homeostasis in patients with diabetes.

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