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miR-184 Regulates Pancreatic β-Cell Function According to Glucose Metabolism*[S]

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Background: Upon entering the pancreatic β-cell, glucose is metabolized to ultimately induce both proliferation and the release of insulin.
Results: *miR-184* targets *Argonaute2* to impact the microRNA pathway according to glucose metabolism.
Conclusion: *miR-184* is a highly regulated microRNA impacting the growth and function of the β-cell.
Significance: These results highlight the adaptive role of the microRNA pathway based on metabolic state.

In response to fasting or hyperglycemia, the pancreatic β-cell alters its output of secreted insulin; however, the pathways governing this adaptive response are not entirely established. Although the precise role of microRNAs (miRNAs) is also unclear, a recurring theme emphasizes their function in cellular stress responses. We recently showed that *miR-184*, an abundant miRNA in the β-cell, regulates compensatory proliferation and secretion during insulin resistance. Consistent with previous studies showing *miR-184* suppresses insulin release, expression of this miRNA was increased in islets after fasting, demonstrating an active role in the β-cell as glucose levels lower and the insulin demand ceases. Additionally, *miR-184* was negatively regulated upon the administration of a sucrose-rich diet in *Drosophila*, demonstrating strong conservation of this pathway through evolution. Furthermore, *miR-184* and its target *Argonaute2* remained inversely correlated as concentrations of extracellular glucose increased, underlining a functional relationship between this miRNA and its targets. Lastly, restoration of *Argonaute2* in the presence of *miR-184* rescued suppression of *miR-375*-targeted genes, suggesting these genes act in a coordinated manner during changes in the metabolic context. Together, these results highlight the adaptive role of *miR-184* according to glucose metabolism and suggest the regulatory role of this miRNA in energy homeostasis is highly conserved.

Our understanding of the compensatory mechanisms controlling pancreatic β-cell function according to changes in extracellular glucose remains incomplete (1, 2). Upon entering the β-cell, glucose is metabolized to ultimately induce both proliferation and the release of insulin (3–5). However, the full extent to which genes that regulate growth and secretion respond to the molecular events that follow glycolysis, including mitochondrial and ATP metabolism, is not completely known. Recent studies have shown that deletion of glucokinase (*Gck*) reduced β-cell replication, underlining the role of glucose as a key mitogenic factor in this cell type (6). In addition, the BCL-2 family member BAD occupies a glucokinase-containing complex to regulate mitochondrial respiration in the β-cell in response to glucose (7). Moreover, in response to a glucose stimulus, quiescent β-cells entered the G_1 phase of the cell cycle further emphasizing the tight association between the glycolytic pathway and the mechanisms regulating β-cell mass (8). Specific cell cycle regulators and polycomb group (PcG) proteins have also been shown to regulate β-cell proliferation and regeneration, indicating that many conserved and functionally diverse factors contribute to this process (9, 10). Furthermore, the growth rate of the β-cell declines with age in both mouse and human, indicating many regulatory mechanisms act in an age-dependent manner (11, 12).

We recently showed that *miR-184*, a highly conserved and abundant miRNA[2] expressed in the β-cell, regulates compensatory proliferation and insulin secretion during insulin resis-

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The NCBI Gene Expression Omnibus (GEO) and Sequence Read Archive (SRA) accession numbers for the referenced array and sequencing data are GSE46623.
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[2] The abbreviations used are: miRNA, microRNA; *Gck*, glucokinase; 184KO, *miR-184* knock-out; dox, doxycycline; qRT, quantitative real-time; SILAC, stable isotope labeling by amino acids in cell culture.

tance (13). Using both genetic and diet-induced mouse models of obesity and insulin resistance, we observed the silencing of *miR-184* in the pancreatic islet, and upon treatment of a low carbohydrate ketogenic diet, its expression was rescued (13). Importantly, these results suggested that miRNAs may respond to changes in the metabolic environment of the cell including systemic insulin sensitivity and glucose concentrations. Although the precise role of the miRNA pathway remains to be established, many studies have highlighted its regulatory role in gene regulation during adaptive response mechanisms (14). Under steady-state conditions, many loss-of-function mouse models for miRNA genes exhibit subtle phenotypes that become more pronounced upon the induction of physiologic stresses (15). To date, the impact of changes in nutrient intake and sensing on the miRNA pathway has not been characterized. Therefore, our main goal was to identify the extent to which miRNAs are altered according to extracellular glucose levels and to determine the functional relevance of their regulation.

In this study we first reinvestigated the impact of a long term ketogenic diet on *miR-184* expression in the pancreatic β-cell; as in our previous work we found that administration of this diet to hyperglycemic *ob*/*ob* mice restored both insulin sensitivity and normoglycemia (13). Furthermore, our studies also show that reverting from a ketogenic diet back to a normal chow restores *miR-184* expression to normal within 24 h, illustrating the modulatory behavior of this one specific miRNA. Moreover, fasting and inhibition of glycolysis both resulted in increased levels of *miR-184*, indicating this miRNA is activated to suppress secretion as the demand for insulin is abated. Lastly, our observations showing the silencing of *miR-184* in response to a high sucrose diet in *Drosophila* suggests this miRNA may contribute to a highly conserved mechanism regulating energy homeostasis. Together these results identify the adaptive functional role of *miR-184* according to glucose metabolism and establish the conservation of its modulatory behavior to *Drosophila*.

Experimental Procedures

Generation and Maintenance of Animals—Mice were maintained on a 12-h light/dark cycle with *ad libitum* access to regular chow food or ketogenic diet (catalog number E15149-30, ssniff Spezialdiäten GmbH) in accordance with requirements established by Landesamt für Gesundheit und Soziales (Lageso). All experimental procedures were approved under protocols G 0357/10, O 0405/09, and T 0436/08. The total *miR-184* knock-out (184KO), *dox-184*, and *dox-Ago2* mice were generated and genotyped as previously described (13).

Gene Expression Array Analysis—MIN6 cells were transfected with rtTA reverse transactivator along with 184-tetO plasmids. Overexpression of *miR-184* was induced by 1 μg/ml doxycycline (Sigma) at time points between 16 and 72 h in triplicate. Cells were harvested, and cDNA synthesis was performed from total RNA using the Illumina TotalPrep RNA Amplification kit (AMIL1791, Life Technologies) and then hybridized using Illumina mouse WG6v2 arrays. Raw data from the Illumina scanner were loaded into R using the lumi package (Illumina). Mappings to gene names and gene IDs were provided by the lumiMouseIDMapping package. Light intensities were quantile-normalized using the lumiN function, and the analysis focused on probes for the detection of p values <0.05 either in the transfection control or at any of the time points of the experiment. For subsequent analyses, we focused on these probes, discarding all others. Mappings of probes to gene IDs were obtained from the lumiMouseAll.db package, and we computed the differential regulation in gene expression as the log 2-fold change in signal intensity at the different time points compared with the transfection control. We investigated the effect of the *miR-184* induction on the *miR-184* target genes as well as on the target genes of *miR-375*, *miR-182*, *miR-30a*, and *148a/152* that are highly expressed in MIN6 cells. For each of these four miRNAs, we collected groups of target genes according to the presence of a canonical binding site in the 3′-UTR, defined as a heptamer complementary to positions 2–8 of the miRNA, or to positions 2–7 with a 'U' at position 1 (16). A fifth group (which we called "no seed") consisted of genes with no canonical binding site for any of these miRNAs in the 3′-UTR. 3′-UTR sequences were downloaded from the RefSeq database (NCBI) on January 18, 2011. For each of these groups of genes and for each time point, we finally computed the mean log 2-fold change in gene expression upon *miR-184* induction as well as the standard error.

Gene Expression Analysis in Drosophila—Canton-s flies were maintained at 25 °C in 12 h light:12 h dark cycles on a standard diet (yeast, 38 g/liter; yellow corn mill, 91 g/liter; agar, 10 g/liter; molasses, 8.7% v/v; propanoic acid (BioLab), 0.9% v/v; Tegasept solution (Sigma; 300 g/liter in EtOH (BioLab)), 0.8% v/v). For experimental manipulation, 3-day-old canton-s flies were starved for 16 h and then supplemented with sucrose (2% agar and 5% sucrose food media), and flies were collected at 0, 6, 12, and 18 h. RNA was generated from fly heads by using TRIzol reagent (Invitrogen). RNA was incubated with poly(A) polymerase (Ambion), and cDNA was synthesized using oligo-dT primers. qRT-PCR was performed with Bio-Rad (C 1000^{TM} Thermal cycler) real time PCR. The following primer sequences were used in gene study: *mir-184* GACGGAGAACTGATAAGGG; ribosomal protein 49 (*rp-49*), CGGTTACGGATCGAACA; universal primer, GCGAGCACAGAATTAATACGAC. miRNA values were normalized with the ribosomal protein 49 (*rp-49*).

Cell Culture, Biochemical Fractionation, and Antibodies—MIN6 cells were cultured in DMEM (Invitrogen) containing 4.5 g/liter glucose supplemented with 15% v/v heat-inactivated FCS, 50 μM β-mercaptoethanol, and 50 mg/ml penicillin, and 100 mg/ml streptomycin. SILAC-labeling of MIN6 cells and LC-MS/MS-based quantitative proteomics were performed exactly the same way as described previously (23). Antibodies that were used throughout this work were as follow: Ago2 (Cell Signaling C34C6), γ-tubulin (Sigma T6557), Rab3a (Abcam ab3335), Nsf (BD Bioscience), Grp78 (Assay Designs StressGen SPA-826), Slc25a22 (Sigma AV44041), Dicer (Bethyl A301–936A), and Bcl-xL (Cell Signaling #2764). For biochemical fractionation, an eight-step sucrose gradient was performed on MIN6 cells as described previously (23). Briefly, MIN6 cells were washed, pelleted, and resuspended in homogenization buffer containing 5 mM HEPES, 0.5 mM EGTA, and 1× Complete Protease inhibitors (Roche Applied Science) at pH 7.4 and

later homogenized. The homogenate was spun at 3000 × *g* for 10 min at 4 °C, and the post-nuclear supernatant was loaded onto an 8-step discontinuous sucrose density gradient (HEPES-buffered 0.2–2 M sucrose) and centrifuged at 55,000 rpm for 2 h at 4 °C using an MLS50 rotor (Beckman Coulter). Mitochondrial subcellular fractionation was performed as described previously with minor modifications (17). Briefly, 1×10^7 MIN6 cells were washed, resuspended in isotonic mitochondrial buffer (250 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES pH 7.5), and homogenized. The lysates were spun at 500 × *g* for 5 min to eliminate unbroken cells, and the supernatant was centrifuged at 10,000 × *g* for 30 min at 4 °C. The mitochondria-enriched pellet was washed twice and resuspended in mitochondrial buffer for downstream Western blotting analysis.

Transmission Electron Microscopy—Approximately 50 isolated islets were fixed in 2.5% glutaraldehyde in Millonig's buffer (2.26% NaH_2PO_4 and 2.52% NaOH) for 2 h at 4 °C and then stained in 1.0% osmium tetroxide for 1 h. After dehydration in ethanol, islets were embedded in AGAR 100 (Oxfors Instruments Nordiska AB, Sweden), sectioned (70–90 nm thick), placed on copper grids, and contrasted with uranyl acetate and lead citrate. Imaging was performed on JEM 1230 electron microscope (JEOL-USA, Inc., MA), and micrographs were analyzed with respect to mitochondrial morphology.

Analytic Procedures—Insulin measurements from plasma and sucrose gradient fractions were measured by radioimmunoassay (Millipore), blood glucose was measured as described (18). *In vivo* glucose or insulin tolerance tests were performed after a 6-h fast and injected intraperitoneally with either glucose (1 g/kg body weight) or insulin (0.75 units/kg body weight), respectively.

Statistical Analysis—All qRT-PCR results are expressed as the mean ± S.E. Comparisons between data sets with two groups were evaluated using an unpaired Student's *t* test. A p value of ≤ 0.05 was considered statistically significant. The correlation plots were performed using GraphPad Prism.

Results

miR-184 Is Regulated According to Glucose Metabolism—*miR-184* is well expressed in the islets when compared with other endocrine tissues such as pituitary, adrenal, and thyroid and more specifically enriched in the MIN6 β-cell line compared with other cell lines of exocrine pancreas (Fig. 1, *A* and *B*). Consistent with our previous observation, long term administration of the ketogenic diet for 75 days to C57BL/6 wild-type mice induced ~3-fold over-expression of *miR-184* in their islets (13) (Fig. 1*C*). In addition, after reverting wild-type mice fed a ketogenic diet for 25 days back to normal chow, *miR-184* expression was restored to normal levels in 24 h (Fig. 1*D*). Moreover, returning mice to a normal chow diet also reversed the effects on both glucose tolerance and insulin sensitivity (Fig. 1, *E* and *F*). To further test the effect of nutrient intake on the expression of this miRNA, we next fasted C57BL/6 mice for 30 h, which lowered both glucose and insulin levels. During this fasting phase, we observed a significant increase in *miR-184* in isolated islets from these animals, and this increase was normalized 24 h after re-feeding the fasted mice (Fig. 1*G*). To test whether circulating factors impacting energy homeostasis contribute to changes in *miR-184* expression, we treated MIN6 cells with either 100 nM insulin or 20 nM exendin-4 (*Ex4*) for 48 h and observed no changes in miRNA expression by qRT-PCR (Fig. 1*H*). In line with fasting, feeding mice with a caloric-restricted diet over a period of 25 days also resulted in an enhanced expression of *miR-184*, whereas the levels of *miR-375* remained unchanged in the isolated islets from these mice (Fig. 1*I*). Moreover, it has been previously shown that *miR-184* was silenced in both the pancreatic islets of type 2 diabetic human subjects compared with non-diabetic controls and in Aplysia sea snails after administration of serotonin in the central nervous system, suggesting its functional role may be conserved a great distance (13, 19). *miR-184* is also abundantly expressed in *Drosophila*, a widely used model for the study of metabolism and longevity; however, its precise functional role in this species remains unknown (20–22). To determine whether *miR-184* is also regulated in response to metabolic stimuli in *Drosophila*, we first provided a sucrose-rich diet to flies after a 16-h fasting period. *miR-184* expression significantly decreased 6 h after initiating this diet, indicating that the silencing of this miRNA as glucose metabolism increases is strongly conserved (Fig. 1*J*). Consistent with our observations in isolated mouse islets, fasting of flies also induced the expression of this miRNA, and upon re-feeding, its levels were normalized (Fig. 1*J*).

miR-184 and Ago2 Remain Inversely Correlated According to Glucose Metabolism—To further understand the role of glucose metabolism in regulating *miR-184*, we next inhibited glycolysis either by siRNA-mediated knockdown of *Gck* or treatment of 2-deoxyglucose in MIN6 cells and observed an increase in the expression of *miR-184* (Fig. 2, *A* and *B*). Conversely, treatment with the glucokinase activator RO-28-1675 resulted in reduced *miR-184* levels consistent with increasing glucose metabolism (Fig. 2*C*). Moreover, the inverse correlation between *miR-184* and its targets was also observed upon incubating both MIN6 cells and isolated islets in high and low glucose concentrations (Fig. 2, *D*–*G*).

Although previous studies have shown that *miR-184* mediates the developmental transition of the female germ line in *Drosophila* as well as neural stem cell differentiation and proliferation in mice, *miR-184* knock-out mice (184KO) did not display developmental or behavioral abnormalities as quantified by body weight, food intake, energy expenditure, and locomotor activity in comparison to littermate controls (Fig. 2, *H*–*K*) (23, 24). To ultimately test the direct role of *miR-184* in maintaining glucose levels, we fasted 184KO mice and observed decreased blood glucose and increased plasma insulin levels (Fig. 2*L*). These results indicate that *miR-184* can directly contribute to systemic glucose levels as glucose metabolism is reduced in the β-cell. Lastly, administration of an insulin tolerance test on *miR-184* transgenic mice (*dox-184*) that overexpress *miR-184* in a doxycycline-inducible manner revealed no changes compared with littermate controls indicating increased expression of this miRNA does not significantly contribute to systemic insulin sensitivity (Fig. 2*M*). Together, our results indicate that it is the regulation of this miRNA in response to changes in energy metabolism that is conserved from mice to flies.

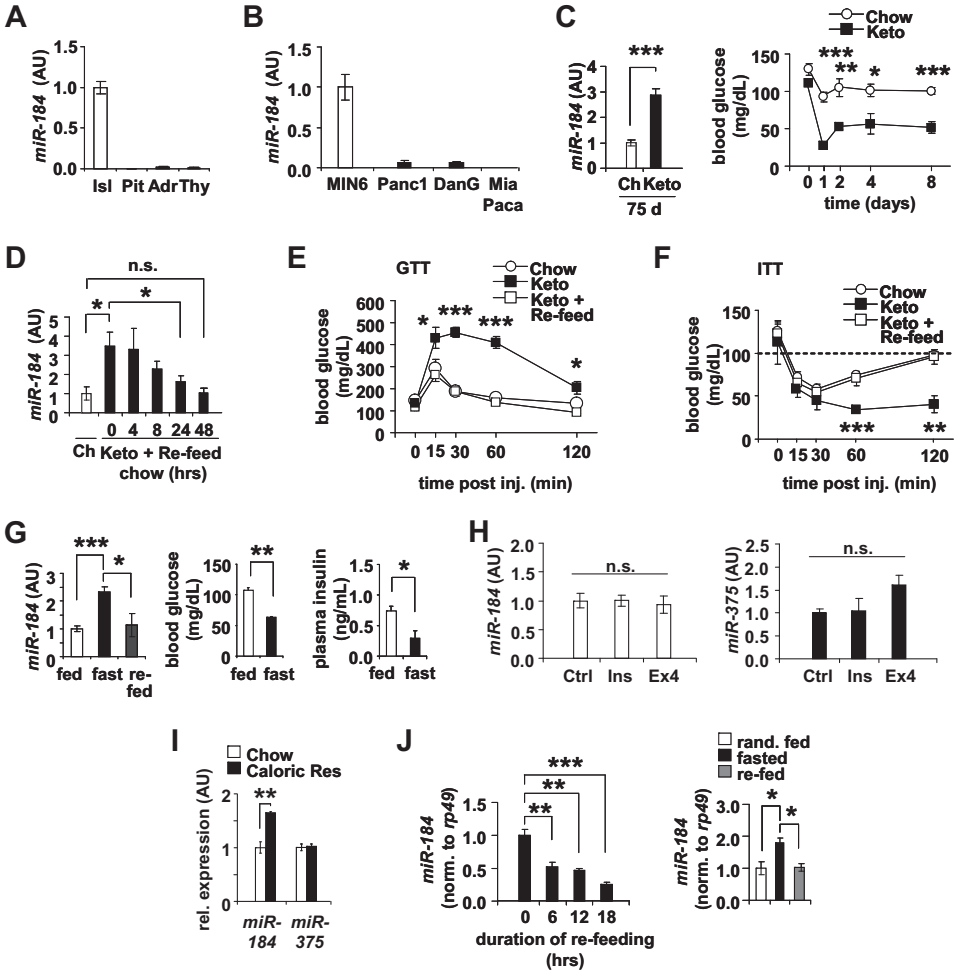


FIGURE 1. ***miR-184* is regulated according to glucose metabolism.** *A*, qRT-PCR analysis of relative *miR-184* expression in isolated pancreatic islets (*Isl*), pituitary (*Pit*), adrenal gland (*Adr*), and thyroid (*Thy*) from 10-week-old C57BL/6 mice. *AU*, arbitrary units. *B*, qRT-PCR analysis of relative *miR-184* expression in several pancreatic cell lines including MIN6, Panc1, DanG, and MiaPaca. *C*, qRT-PCR analysis of *miR-184* in isolated islets of 10-week-old *WT* mice on either a normal chow (*Ch*) or ketogenic (*Keto*) diet for 75 days ($n = 4$). Random blood glucose levels were decreased after 1 day on ketogenic diet. Results are presented as mean ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. *D*, qRT-PCR analysis of *miR-184* in isolated islets of 10-week-old WT mice on either normal chow or ketogenic diets for 25 days. Mice on the ketogenic diet were reverted to normal chow for 0–48 h ($n = 4$). *n.s.*, not significant. *E*, blood glucose levels during a glucose tolerance test (*GTT*) from 10-week-old C57BL/6 mice on normal chow (*Chow*), ketogenic diet (*Keto*), and normal chow after 25 days on ketogenic diet (*Keto + re-feed*) ($n = 4$). *F*, blood glucose levels during an insulin tolerance test (*ITT*) from 10-week-old C57BL/6 mice on normal chow (*Chow*), ketogenic diet (*Keto*), and normal chow after 25 days on ketogenic diet (*Keto + re-feed*) ($n = 4$). *G*, qRT-PCR analysis of *miR-184* and *miR-375* in isolated islets of 10 weeks old WT mice fasted for 24 h. Fed and fasted blood glucose and insulin parameters measured before sacrifice ($n = 4$). *H*, qRT-PCR analysis of *miR-184* and *miR-375* after treating MIN6 cells with either 100 nM insulin or 20 nM of exendin-4 (*Ex4*) for 48 h ($n = 3–4$). *I*, qRT-PCR analysis of *miR-184* in isolated islets of 10-week-old WT mice on either normal chow or a low calorie diet for 25 days ($n = 4$). *J*, qRT-PCR analysis of *miR-184* in *Drosophila* after sucrose administration and after fasting and re-feeding. After a 16 h starvation period, flies were fed with food in which the only caloric supplement is sucrose (5%). Fly heads were collected from flies re-fed for 0, 6, 12, and 18 h of sugar administration.

miR-184 Is Regulated upon Inhibition of Glucose Metabolism by Tunicamycin—To further test whether inhibition of glucose metabolism by other mechanisms can impact the expression of *miR-184* and its targets, we treated MIN6 cells with the *N*-linked glycosylation blocker, tunicamycin (25). At all concentrations of a dose-response curve, *miR-184* expression was significantly increased compared with vehicle alone, as was the expression of the endoplasmic reticulum stress-related genes, *C/ebpβ* and *Chop* (Fig. 3, *A* and *E*). Again consistent with Ago2 and Slc25a22 being targeted by *miR-184*, the target gene expression decreased after administration of tunicamycin compared with vehicle alone (Fig. 3*B*), whereas the expression of Ago1 and other miRNAs including *miR-375* and *let-7c* did not change (Fig. 3, *C* and *D*). The robustness of the inverse correlation between *miR-184* and its target *Ago2* in response to changes in glucose metabolism suggests an important role for these genes in the maintenance of β-cell function. Although treatment of the MIN6 cells with palmitic acid also induced expression of endoplasmic reticulum stress-related genes, neither *miR-184* nor *Ago2* levels were affected, further supporting the effect of glucose metabolism on their expression (Fig. 3*F*). Together, these results strongly implicate the regulation of *miR-184* and its targets according to glucose metabolism.

miR-184 Regulates the β-Cell Secretome—We further addressed the impact of *miR-184* on β-cell function by quantifying the secreted proteins after overexpression of this miRNA. SILAC-labeled MIN6 cells that were transfected with *miR-184* mimics exhibited a reduced capacity to secrete the previously identified cluster of proteins upon receiving high glucose (Fig. 4*A* and supplemental Table S1) (26). Importantly, a significant

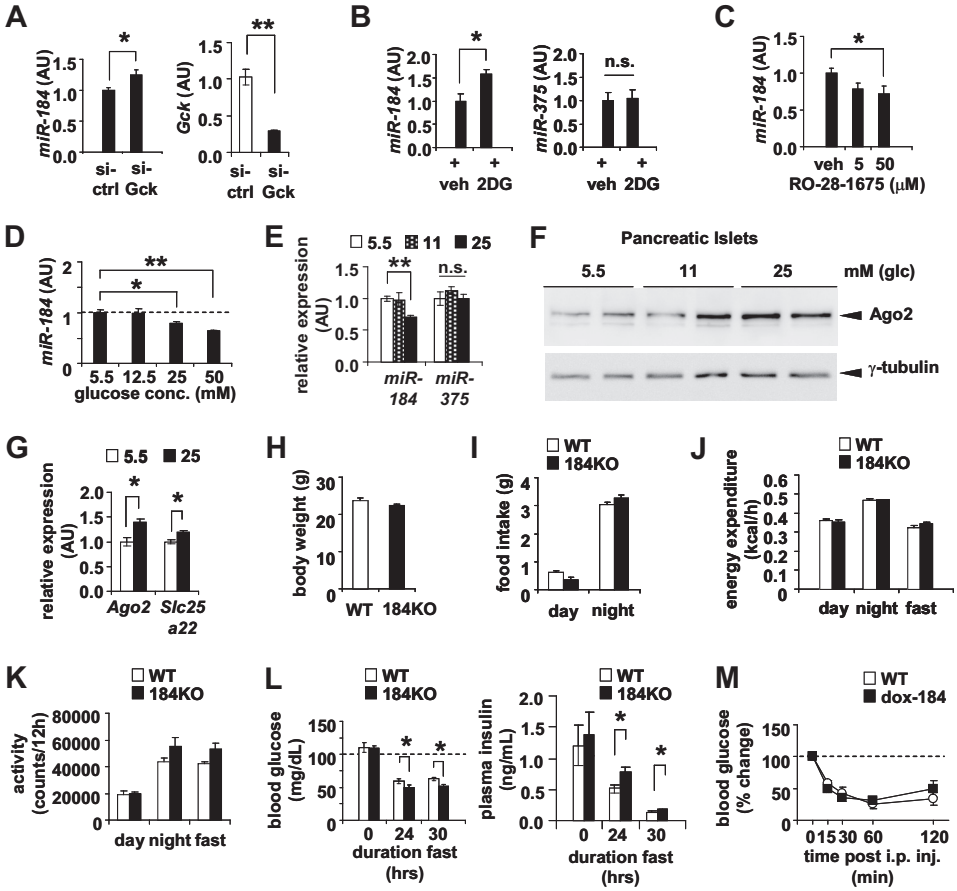


FIGURE 2. ***miR-184* and Ago2 remain inversely correlated according to glucose metabolism.** *A*, qRT-PCR analysis of *miR-184* in MIN6 cells transfected with either 200 pmol of siRNA targeting glucokinase (*si-Gck*) or scrambled control (*si-Ctrl*) for 48 h. *AU*, arbitrary units. *B*, qRT-PCR analysis of *miR-184* and *miR-375* in MIN6 cells after treatment with 2-deoxyglucose (*2DG*) or vehicle control (*veh*) for 48 h. *C*, qRT-PCR analysis of *miR-184* in MIN6 cells after receiving 5 or 50 μm glucokinase activator (RO-28–1675) or vehicle control (*veh*) for 48 h. *D*, qRT-PCR analysis of *miR-184* in MIN6 cells after treatment of 5.5, 12.5, 25, and 50 mM of glucose for 48 h ($n = 4$ for all concentrations). *E*, qRT-PCR analysis of *miR-184* and *miR-375* in isolated islets of 10-week-old WT mice that were treated *ex vivo* with either 5.5, 11, or 25 mM glucose ($n = 4$). *n.s.*, not significant. *F*, Western blotting analysis of Ago2 in the isolated islets of 10-week-old WT mice that were treated *ex vivo* with 5.5, 11, or 25 mM glucose. *G*, qRT-PCR analysis of *Ago2* and *Slc25a22* in isolated islets treated *ex vivo* with either 5.5 or 25 mM glucose ($n = 4$). *H*, body weight measurements of 10-week-old 184KO mice compared with their WT littermate controls ($n = 4$). *I*, food intake measurements of 10-week-old 184KO mice compared with their WT littermate controls during day and night ($n = 4$). *J*, energy expenditure measurements of 10-week-old 184KO mice compared with their WT littermate controls during the day and night ($n = 4$). *K*, activity measurements of 10-week-old 184KO mice compared with their WT littermate controls during the day and night ($n = 4$). *L*, fasted blood glucose and plasma insulin levels of 10-week-old 184KO mice compared with their littermate controls ($n = 4–6$). *M*, blood glucose levels during an insulin tolerance test (*ITT*) from 8-week-old WT and *dox-184* mice ($n = 3–4$). Results presented as mean ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

overlap was observed after treatment with *miR-184* mimics compared with previous secretome profiles that were identified after high glucose treatment or siRNA-mediated inhibition of *glucokinase* (Fig. 4, *B–D*) (26). These observations are consistent with previous results after overexpression of *miR-375*, indicating several miRNAs may coordinately contribute to the mechanisms leading up to the release of insulin by the β-cell and not to the composition of insulin-containing granules (26). As none of the proteins identified as a part of the secretome is predicted as direct targets of *miR-184*, these results may suggest abundant miRNAs may impact glucose sensing, mitochondrial metabolism or localization, ion channel function, or the recruitment or fusion of vesicles to the plasma membrane.

miR-184 Regulates Mitochondrial Respiration—We previously identified the mitochondrial gene *Slc25a22* as a target of *miR-184* and, therefore, investigated the effect of this miRNA on cellular respiration (13). Transfection of *miR-184* mimics in MIN6 cells resulted in a significant decrease of mitochondrial coupling efficiency, and this reduction was caused by reduced glucose-stimulated cellular respiration and not altered mitochondrial proton leak (Fig. 5, *A* and *B*). Because the basal respiration remained unchanged, these observations indicate that *miR-184* regulates secretion via mitochondrial substrate flux in the β-cell, possibly by suppressing validated targets such as *Slc25a22* at the mitochondria (Fig. 5, *C* and *D*) (13). Furthermore, transmission electron microscopic analysis of the islets isolated from *dox-184* mice revealed a number of morphological alterations including disruption of the lamellae within the mitochondria (Fig. 5, *E* and *F*) (13). Previous studies have associated these changes with the loss of cytochrome *c*-4CYS and mitochondrial function (27). Additionally, a small reduction in the number of mitochondria per cell was observed in *dox-184* β-cells ($p = 0.10$, quantified from transmission electron microscopy images at 8000 magnification: mean (WT) = 22.5 mitochondria/cell; mean (*dox-184*) = 18.5 mitochondria/cell; 25 cells from each set pooled from $n = 3$ mice). Together these results suggest

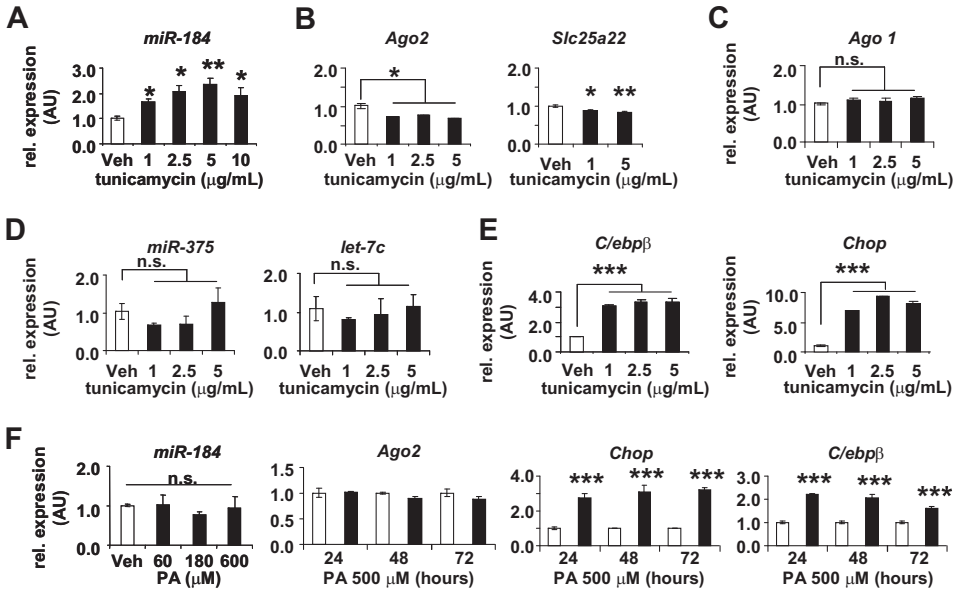


FIGURE 3. ***miR-184* is regulated upon inhibition of glucose metabolism by tunicamycin.** qRT-PCR analysis of *miR-184* (*A*) *Ago2* and *Slc25a22* (*B*), *Ago1* (*C*), *miR-375* and *let-7c* (*D*), and *C/ebpβ* and *Chop* (E) mRNA expression in MIN6 cells treated with increasing concentrations of tunicamycin for 48 h ($n = 4$ for each concentration). *AU*, arbitrary units; *Veh*, vehicle. *n.s.*, not significant. *F*, qRT-PCR analysis of *miR-184* in MIN6 cells treated with increasing concentrations of palmitic acid (*PA*) for 48 h and the levels of *Ago2*, *C/ebpβ*, and *Chop* expression measured after treating MIN6 cells with 500 μM PA over time ($n = 4$ for each concentration and time point). Results are presented as the mean ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

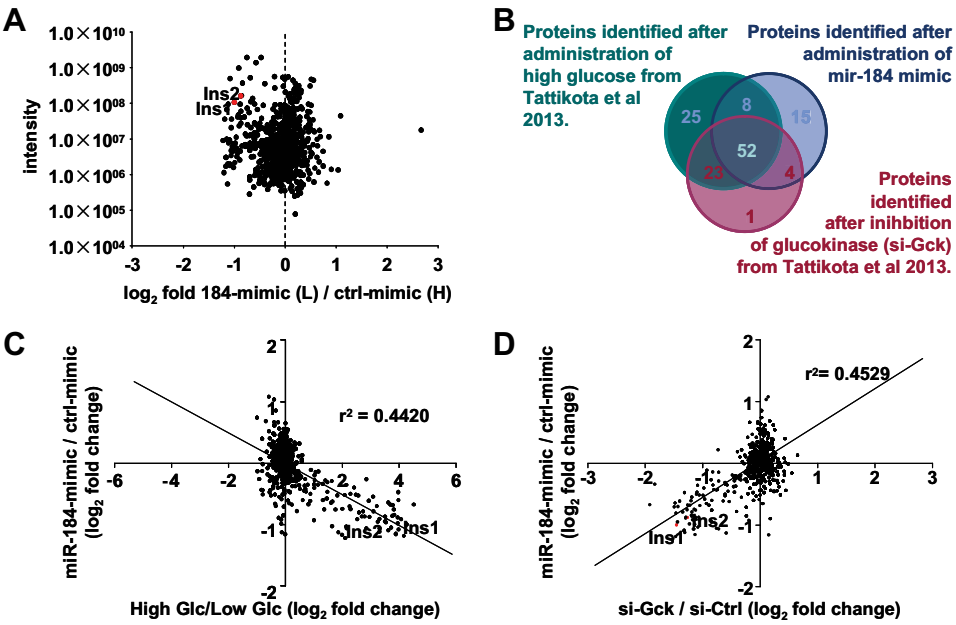


FIGURE 4. ***miR-184* regulates the β-cell secretome.** *A*, identification of secreted factors that were inhibited after a high glucose stimulus of 184-mimic-transfected "light" compared with Ctrl-mimic transfected "heavy" MIN6 cells after SILAC labeling. *B*, Venn diagram representation of the secreted factors of MIN6 cells transfected with 184-mimic (from the current study) and from MIN6 cells treated with high/low glucose and si-*Gck* (taken from Tattikota *et al.* (26)) having a cutoff value of 0.5 log 2-fold change. *C* and *D*, intersection of the secretome profiles of MIN6 cells transfected with 184-mimic (from the current study) and of MIN6 cells treated with high/low glucose and si-*Gck* (taken from Tattikota *et al.* (26)) displays an inverse and positive correlation, respectively.

that *miR-184* may impact the β-cell secretome by targeting *Slc25a22* and ultimately mitochondrial respiration.

Ago2 Is Localized at the Mitochondria in MIN6 Cells—As recent studies have established a role for Ago2 within the mitochondria (28), we next examined the localization of Ago2 in MIN6 cells. Ago2 was found abundant in eluted fractions independent of the insulin peak and the granule markers Nsf and Rab3a, suggesting it is not enriched at the secretory granules (Fig. 6*A*). Western blotting identified Ago2, Slc25a22, and Bcl-xL were present in the mitochondria-containing fractions after subcellular purification (29, 30) (Fig. 6*B*). siRNA-mediated knockdown of Ago2 in MIN6 cells further confirmed its expression in both the cytoplasmic and mitochondrial fractions (Fig. 6*C*). These data suggest that *miR-184* may impact the growth and function of the β-cell by targeting genes present at the mitochondria including Ago2 and Slc25a22.

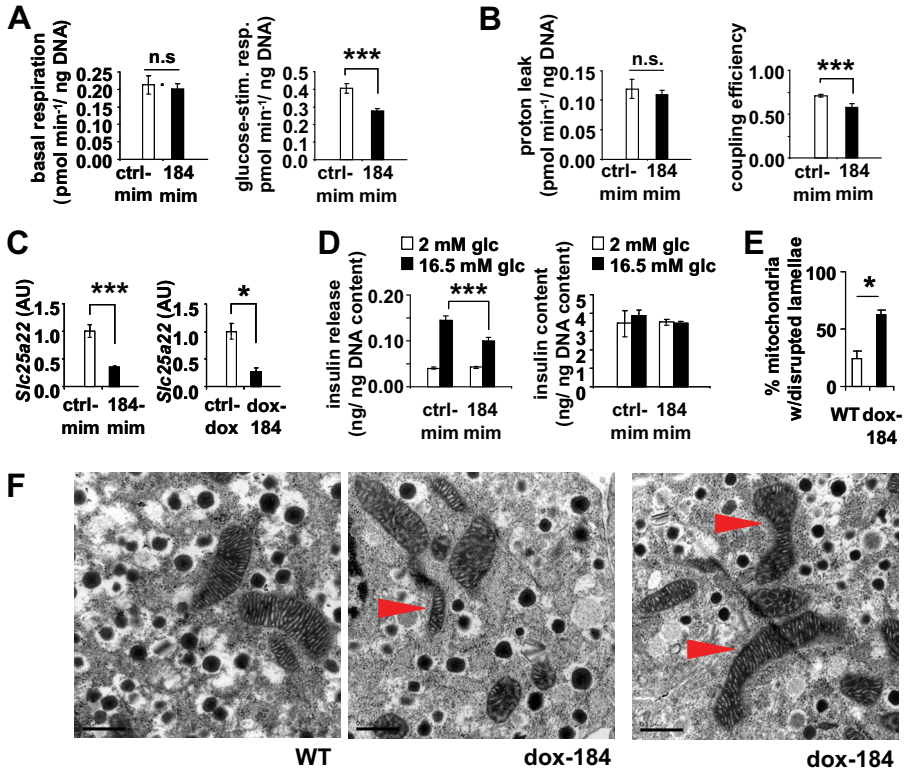


FIGURE 5. ***miR-184* impacts mitochondrial respiration.** Measurements of basal respiration and glucose-stimulated respiration (*A*), proton leak and mitochondrial coupling efficiency ($n = 3$) in MIN6 cells transfected with either control or 184-mimic (*B*), and qRT-PCR analysis of *miR-184*-targeted gene *Slc25a22* in MIN6 cells transfected with either control or 184-mimic and in the islets of *dox-184* transgenic mice (*C*), respectively. *n.s.*, not significant; *AU*, arbitrary units. *D*, reduced glucose-stimulated insulin secretion in MIN6 cells transfected with either control or 184-mimic. Results are presented as the mean ± S.E. *, $p < 0.05$; ***, $p < 0.001$. *E*, analysis of mitochondria from the beta cells within the islets isolated from WT *ctrl-dox* and *dox-184* mice that received 1 mg/ml doxycycline for 15 days. Data are represented as the percentage of mitochondria with disrupted lamellae. *F*, transmission electron microscopic (*TEM*) images of the beta cells within the islets from WT and *dox-184* mice that received 1 mg/ml doxycycline for 15 days. Scale bar 0.5 μm.

Restoration of Ago2 in the Presence of miR-184 Maintains Normal Glucose Homeostasis—To investigate the impact of altering expression of *miR-184* on the β-cell transcriptome, we performed gene expression analysis using Illumina mouse W2vG arrays. The murine MIN6 β-cell line was transfected with two plasmids expressing 1) the reverse tetracycline transactivator (rtTA) and 2) a genomic fragment encompassing the *miR-184* precursor under the control of the tet operon (*184-tetO*). Upon treating the cells with 1 μg/ml doxycycline (dox), we observed a progressive increase of *miR-184* after 16 h of dox administration, whereas genes containing *miR-184*-seed matches significantly decreased their expression compared with genes without seed matches (Fig. 7, *A* and *B*). The down-regulation of *miR-184* targets was transient over time, in line with *miR-184* targeting Ago2. In addition, genes containing matches for the seeds of three additional miRNAs that are abundant in this cell line including *miR-375*, *182*, *30a*, and *148a/152*, were progressively up-regulated compared with genes without seed matches for these miRNAs, consistent with the idea that *miR-184* can impact miRNA-mediated regulation via the down-regulation of Ago2 (Fig. 7*A*).

To further test the physiologic relevance of the targeting of Ago2 by *miR-184*, we next crossed two previously generated transgenic mouse models that allowed overexpression of *Ago2* and *miR-184* in the pancreatic β-cell in a doxycycline-inducible manner (*dox-Ago2* and *dox-184*, respectively) (13). Doxycycline-dependent Ago2 expression was possible in the presence of *miR-184* in *dox-184/Ago2* animals as the Ago2 transgene lacked the 3′-UTR harboring the *miR-184*-seed sequence (13). *Dox-184/Ago2* mice exhibited comparable expression of both *miR-184* and Ago2 to their littermate controls, *dox-184* and *dox-Ago2*, respectively (Fig. 7, *C* and *D*). Although *dox-Ago2* mice displayed normal random glucose and glucose tolerance after treatment of doxycycline, *dox-184* animals were hyperglycemic as a result of decreased circulating insulin as previously shown (Fig. 7, *E–G*) (13). Interestingly, *dox-184/Ago2* mice maintained normal steady-state glucose and insulin levels and glucose tolerance in contrast to *dox-184* mice, indicating that the maintenance of Ago2 expression in the presence of *miR-184* restored normal glucose control (Fig. 7, *F–H*). Furthermore, pancreatic β-cell mass and proliferation rate were also restored in *dox-184/Ago2* mice consistent with the maintenance of glucose homeostasis in these animals (Fig. 7, *I* and *J*) (13). To determine whether the capacity to maintain miRNA-mediated gene regulation by Ago2 remained intact in *dox-184/Ago2* mice, we next assessed the expression of *miR-375*-targeted genes in isolated islets. Expression of *Cadm1*, *Gphn*, and *Rasd1* was lower in islets isolated from *dox-184/Ago2* mice and islets of *dox-Ago2* animals compared with littermate controls (Fig. 7*K*). Although the mechanism of this rescue is not precisely clear, these results show Ago2-mediated gene regulation is maintained in these animals and may indicate the function of

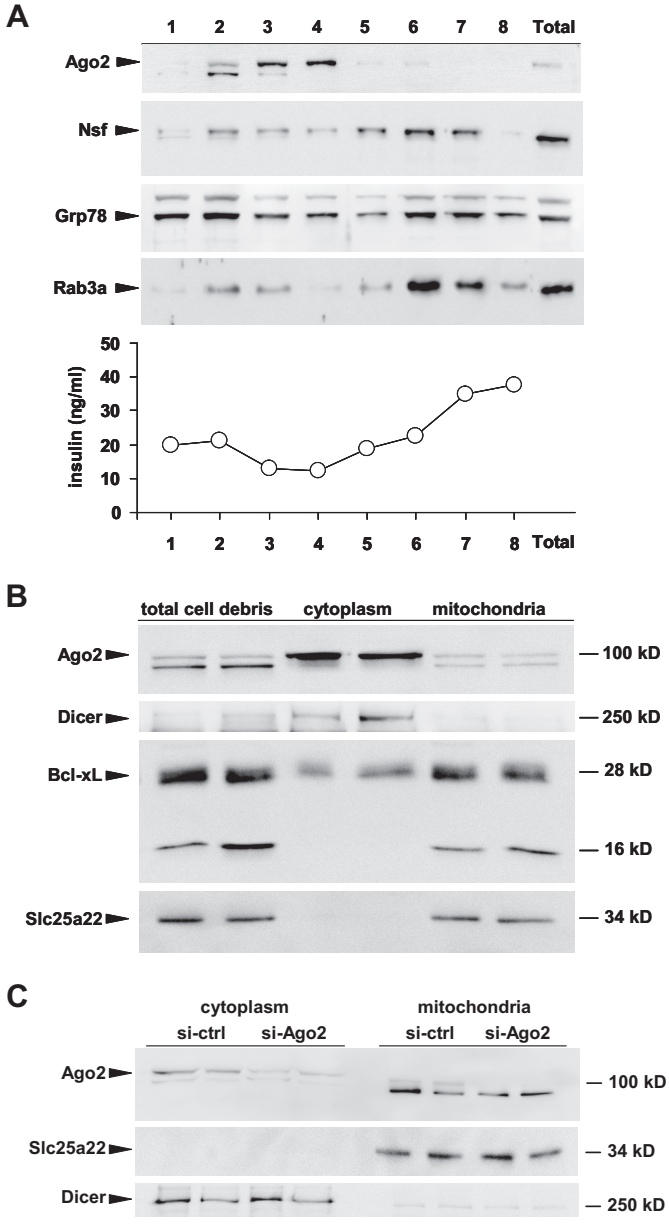


FIGURE 6. **Localization of Ago2 at the mitochondria in MIN6 cells.** *A*, Western blotting analysis and insulin quantification by radioimmunoassay of the fractions derived from a discontinuous sucrose gradient of MIN6 cells. *B*, Western blotting analysis of cytoplasmic and mitochondrial fractions for Ago2, Bcl-xL, Dicer, and Slc25a22. *C*, Western blotting analysis of cytoplasmic and purified mitochondria for Ago2, Dicer, and Slc25a22 after si-RNA mediated knockdown of Ago2 in MIN6 cells.

other abundant miRNAs including *miR-375* countering the action of *miR-184*. To determine whether overexpression of *miR-184* leads to de-differentiation of the β-cell, a broad range of islet marker genes was quantified by qRT-PCR (Fig. 7*L*). Although marked increases in *Ngn3*, *Ppy*, and *Gck* were observed in islets from *dox-184* mice, suggesting a degree of de-differentiation, the majority of genes remained unchanged, indicating *miR-184* is not a significant contributor to β-cell differentiation (Fig. 7*L*).

Discussion

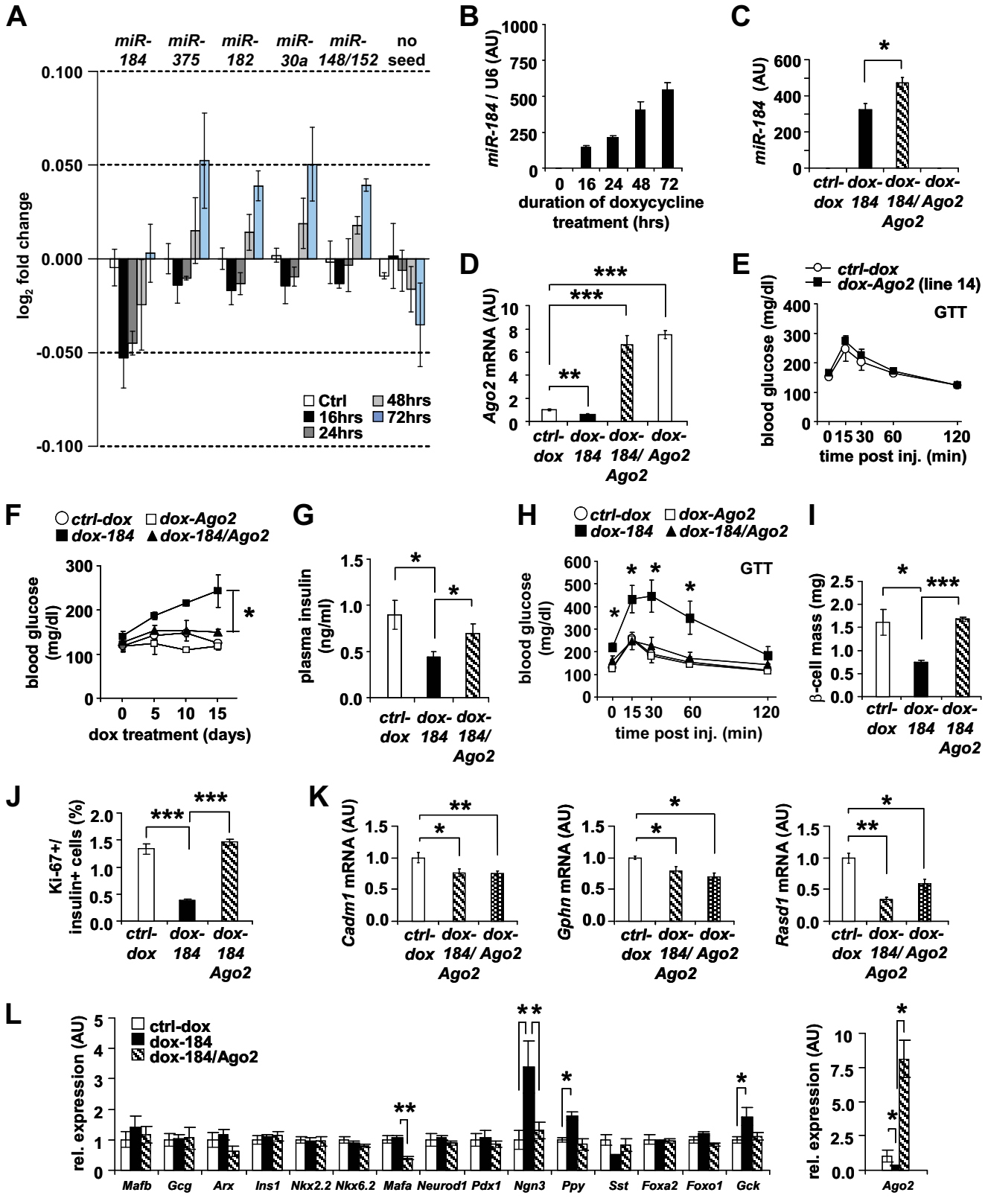
Animals adapt their physiology to changes in nutrient intake through specific molecular mechanisms. Interestingly, organisms at all levels of complexity display increased longevity in response to caloric restriction, suggesting that the fundamental components of these pathways is highly conserved (31, 32). During fasting conditions, it has long been known that the pancreatic β-cell reduces its output of secreted insulin, indicating a central role for glucose metabolism in β-cell physiology (1). We recently showed that *miR-184*, a highly conserved and abundant miRNA expressed in the β-cell, regulates compensatory proliferation and secretion during insulin resistance (13). In contrast to all other miRNAs that are expressed in this cell type, *miR-184* was silenced with the onset insulin resistance and then re-emerged upon administration of a low carbohydrate, ketogenic diet. These observations, after administration of either high fat or ketogenic diets, implicate changes in nutrient intake and insulin sensitivity in the regulation of this miRNA.

Here in this study we follow up on these observations to address the specific impact of nutrient metabolism on the miRNA pathway and show that expression of both *miR-184* and its target *Ago2* are sensitive to changes in glucose metabolism in the β-cell. Although *miR-184* levels in mouse islets increased after a ketogenic diet, the levels of this miRNA were restored within 24 h after reversion to a normal chow diet. In contrast, placing mice on a high fat diet required several weeks for inducing the silencing of this miRNA (13). These results suggest that the β-cell may respond more acutely to specific stimuli such as glucose than to changes in insulin sensitivity. Although the knockouts of *miR-184* and *Ago2* in the β-cell both exhibit no changes during an insulin tolerance test, these results indicate the miRNA pathway in this cell type may not significantly contribute to systemic insulin sensitivity (13, 18). The functional link between *miR-184* and glucose metabolism in the β-cell is further supported by the relatively specific expression profile of this miRNA. In contrast to other abundant miRNAs in the β-cell such as *miR-375*, *miR-184* does not appear to be expressed in the pituitary, adrenal gland, or exocrine pancreas. This limited expression profile may highlight the importance of this miRNA in the regulation of β-cell function according to changes in energy homeostasis.

Importantly, it has long been known that haploinsufficiency of *Gck* resulted in decreased insulin release, underlining the essential role of glucose sensing and metabolism in mediating secretion by the β-cell (33). Our observation that the knockdown of glucokinase resulted in increased expression of *miR-184* indicates that the β-cells activate the expression of this miRNA to suppress insulin release when either glucose sensing or glycolysis is attenuated. Both 2-deoxyglucose and tunicamycin treatments had similar effects, further underlining the role of glucose metabolism in the regulation of *miR-184*. Importantly, although both of these reagents are also widely known to induce endoplasmic reticulum stress and the unfolded protein response, the absence of any effect of palmitic acid suggests that the endoplasmic reticulum stress pathway does not significantly contribute to changes in *miR-184* expression (34).

The precise role of the miRNA pathway remains to be established; however, a recurring theme in many published studies is its function in adaptive stress responses (14). In light of the robust inverse correlation between *miR-184* and *Argonaute2* expression, our observations from the *dox-184/Ago2* mice

would also suggest that many aspects of the involvement of these two genes in glucose homeostasis remain to be described. It is unclear whether the normoglycemia and glucose tolerance observed in these mice results from Ago2-mediated gene silencing or a non-canonical effect of Ago2 on gene expression via its localization in the nucleus, mitochondria, or stress granule of the β-cell (28, 35, 36). Nonetheless, the restoration of normal glucose homeostasis and β-cell mass in the *dox-184/Ago2* model provides further support for Ago2 as a biologically relevant target of *miR-184* using an *in vivo* system.



Although our results continue to underline *miR-184* as a potent inhibitor of insulin release, the precise actions of its identified targets remain unclear (13, 26). Inhibition of the two validated targets of this miRNA, *Ago2* and *Slc25a22*, result in contradictory effects on glucose-stimulated insulin secretion and may suggest a hierarchical presence among targets. Although *miR-184* expression is elevated as glucose metabolism is attenuated in the β-cell, it is possible the suppression of Slc25a22 under these conditions may have a more appreciable effect than the suppression of Ago2 in the absence of any glucose stimulus. Conversely, we observed the silencing of *miR-184* in the presence of high extracellular glucose concentrations. Although increased expression of both *Ago2* and *miR-375* will contribute to suppress secretion, these genes mediate compensatory proliferation as metabolic demand increases and may highlight another functional hierarchy within this cell type (13, 18). In light of the proliferative effect of glucose on the β-cell, our results continue to indicate the miRNA pathway facilitates growth and in turn compromises insulin release. It is possible that as the need for insulin rises, proliferation is prioritized and the suppression of secretion is ultimately inconsequential as more cells are present to alleviate metabolic demand. Importantly, it remains to be precisely described how the β-cell mediates the energy balance between cell size and growth, granule synthesis, and insulin release.

Interestingly, recent studies have shown Ago2 may complex miRNAs independent of target mRNAs according to metabolic state (38). Future studies may identify the majority if not the full extent of β-cell transcripts critical to the regulation of its growth and function complexed to miRNAs and RNA-binding proteins such as Ago2 largely during periods of increased metabolic demand such as the post-prandial state. The occupancy of these β-cell genes by RNA-protein complexes may in turn be determined by metabolic cues such as shifts in systemic glucose levels (39). The presence of miRNAs and RNA-binding proteins on these β-cell genes may act to "stall" their expression until the demand ceases, at which time miRNAs and associated binding proteins are "de-recruited" to an inactive and uncomplexed state. The miRNA pathway may ultimately act as an energy-efficient means of modulating gene expression according to changes in metabolic demand rather than solely implementing *de novo* transcription to promote expression or degradation of critical mRNAs to silence genes.

As *miR-184* is highly conserved, future studies may also address how this miRNA impacts energy homeostasis in other model organisms including *Aplysia* and *Drosophila* to decipher the most fundamental aspects glucose metabolism (22). Although progress is currently hindered due in part to the absence of accurate annotations of orthologous genes between *Drosophila* and mammalian species, several key components of the insulin/Igf-like signaling (IIS) pathway are conserved. In addition, the transcription factor dFOXO has been implicated in metabolic processes in many organisms; however, the extent to which its function is conserved between species remains unclear (37). Likewise, Ago1 is the established mediator of miRNA-mediated gene regulation in *Drosophila* species, further suggesting that although the functional role of *miR-184* may be conserved between species, the transcription factors promoting its expression as well as its direct targets may not be.

Future studies emphasizing the identification of the key factors regulating energy homeostasis, which are conserved between species, will have strong implications on the study of longevity and metabolic disease. Our results shown using *Drosophila* may play a key role in elucidating the fundamental relationship between *miR-184* and energy homeostasis and how these genes can impact the aging process or the onset of insulin resistance and diabetes.

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FIGURE 7. **Restoration of Ago2 in the presence of *miR-184* maintains normal glucose homeostasis.** *A*, Illumina-based gene expression array analysis of the targets of *miRs-184*, *-375*, *-182*, and *-30a* in MIN6 cells overexpressing *miR-184* after 16, 24, 48, and 72 h of doxycycline induction compared with controls (n = 406, 1087, 1575, 1727, and 6679 for *miR-184*, *-375*, *-182*, *-30a*, *148a/152*, respectively, and no seed). *AU*, arbitrary units. *B*, *miR-184* overexpression in MIN6 cells induced by 1 μg/ml doxycycline between 0 and 72 h. *C*, qRT-PCR analysis of *miR-184* in isolated islets of 12-week-old *dox-184*, *dox-184/Ago2*, and *dox-Ago2* transgenic mice compared with their WT *ctrl-dox* littermates treated with 1 mg/ml doxycycline in their drinking water for 15 days (n = 4–6). *D*, qRT-PCR analysis of *Ago2* mRNA in isolated islets of 12-week-old *dox-184*, *dox-184/Ago2*, and *dox-Ago2* transgenic mice compared with their WT *ctrl-dox* littermates treated with 1 mg/ml doxycycline in their drinking water for 15 days (n = 4–6). *E*, blood glucose levels during a glucose tolerance test (*GTT*) on 12-week-old *dox-Ago2* and WT *ctrl-dox* littermates that were not treated with doxycycline (n = 4). *F*, random blood glucose levels of 12-week-old mice compared with *dox-184*, *dox-184/Ago2*, and *dox-Ago2* transgenic mice compared with their WT *ctrl-dox* littermates treated with 1 mg/ml doxycycline in their drinking water for 15 days (n = 12). *G*, random insulin levels of 12-week-old *dox-184* and *dox-184/Ago2* and WT *ctrl-dox* littermates treated with 1 mg/ml doxycycline in their drinking water for 15 days (n = 12–14). *H*, blood glucose levels during a glucose tolerance test on 12-week-old *dox-184*, *dox-184/Ago2*, and *dox-Ago2* transgenic mice compared with their WT *ctrl-dox* littermates treated with 1 mg/ml doxycycline in their drinking water for 15 days (n = 4–6). *I*, quantification of β-cell mass in 12-week-old *dox-184*, *dox-184/Ago2* mice compared with WT *ctrl-dox* littermates treated with 1 mg/ml dox in their drinking water for 15 days (n = 3). *J*, quantification of Ki-67+ β-cells in 12-week-old *dox-184*, *dox-184/Ago2* mice compared with WT *ctrl-dox* littermates (n = 3). *K*, qRT-PCR analysis of *miR-375* targeted genes *Cadm1*, *Gphn*, and *Rasd1* in isolated islets of 12-week old *dox-184*, *dox-184/Ago2*, and *dox-Ago2* transgenic mice compared with their WT *ctrl-dox* littermates treated with 1 mg/ml doxycycline in their drinking water for 15 days (n = 6). *L*, qRT-PCR analysis of islet-specific marker genes in isolated islets of 12-week-old *dox-184*, *dox-184/Ago2* transgenic mice compared with their WT *ctrl-dox* littermates treated with 1 mg/ml doxycycline in their drinking water for 15 days (n = 6). Results are presented as mean ± S.E. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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Gene Regulation:

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