Glucose stimulates microRNA-199 expression in murine pancreatic β-cells

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MicroRNA 199 (miR-199) negatively impacts pancreatic β-cell function and its expression is highly increased in islets from diabetic mice as well as in plasma of diabetic patients. Here we investigated how miR-199 expression is regulated in β-cells by assessing expression of miR-199 precursors (primiR-199a1, primiR-199a2, and primiR-199b) and mature miR-199 (miR-199-3p and miR-199-5p) and promoter transcriptional activity assays in mouse islets and mouse insulinoma cells (MIN6) under different stimuli. We found that mouse islets equally express miR-199-3p and miR-199-5p. However, the primiRNA expression levels differed; although primiR-199a1 expression was about 30% greater than that of primiR-199a2, primiR-199b is barely detected in islets. We observed a 2-fold increase in primiR-199a1 and primiR-199a2 mRNA levels in mouse islets cultured in 10 mM glucose compared with 5.5 mM glucose. Similar responses to glucose were observed in MIN6 cells. Exposure to 30 mM KCl to induce membrane depolarization and calcium influx increased expression of primiR-199a2 but not of primiR-199a1 in MIN6 cells, indicating that calcium influx was involved. Transcriptional activity studies in MIN6 cells also revealed that primiR-199a2 promoter activity was enhanced by glucose and reduced by 2-deoxy-D-glucose–induced starvation. KCl and the potassium channel blocker tolbutamide also stimulated primiR-199a2 promoter activity. Calcium channel blockade by nifedipine reduced primiR-199a2 promoter activity in MIN6 cells, and diazoxide-mediated calcium influx inhibition blunted glucose up-regulation of miR-199-3p in islets. In conclusion, we uncover that glucose acutely up-regulates miR-199 family expression in β-cells. Glucose metabolism and calcium influx are involved in primiR-199a2 expression but not primiR-199a1 expression.

Type 2 diabetes (T2D)[2] is a multifactorial disease and a major public health concern worldwide, triggered by genetic predisposition, environmental cues, or a combination of both (1). Genetic studies support the concept that other factors beyond genetics play a fundamental role in diabetogenesis because only 10% of the overall estimated T2D risk is predicted by gene variants (2–4). Therefore, research in the field turned to exploring other mechanisms that could potentially alter the susceptibility to diabetes. During recent years, there has been increased evidence of a role for noncoding microRNAs (miRNAs) in diabetes. However, how the expression of these miRNAs is regulated and how the diabetes milieu regulates miRNA expression is incompletely understood. In addition, less is known about the molecular mechanisms that regulate the expression of miRNAs during normal or stress conditions and the role of miRNAs in modulation of signaling mechanisms.

miRNAs are ~18- to 22-nucleotide-long noncoding RNAs that suppress the expression of genes by mRNA degradation, mRNA deadenylation, and/or translational repression (5, 6). A single miRNA has multiple target genes, causing widespread changes in protein synthesis for thousands of genes. Therefore, they are key regulators of cell and tissues adaptation to physiological and pathological states. It is predicted that more than 20% of human genes are regulated by miRNAs (7). They are located either intergenically or within a host gene and are transcribed by RNA polymerase II (8, 9). Primary miRNA (primiRNAs) are processed in the nucleus into its precursor (premiRNA) by Drosha and into mature miRNA by Dicer in the cytoplasm (10–12). Then mature miRNAs associate with Argonaute (Ago) proteins in an RNA-induced silencing complex that binds to target sequences generally located in the 39 UTRs of mRNAs, leading to translational repression and/or degradation of the targeted mRNA sequence (13).

The miR-199 family has important and comprehensive functions in different models (14). The family is comprised of the precursors (genes) primiR-199a1, primiR-199a2, and primiR-199b, and they are encoded within introns of the dynamin (DNM) genes in the opposite orientation. In humans, primiR-199a1 is expressed within DNM2 in chromosome (Chr) 19, primiR-199a2 is expressed within DNM3 in Chr3, and primiR-199b is within DNM1 in Chr9 (15). In mice, primiR-199a1, primiR-199a2, and primiR-199b are located in Chr9, Chr1, and Chr2, respectively (14). There is no evidence of functional correlation between expression of the dynamin genes and primiR-199, indicating that expression of the miRNA precursors is con-

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[2] The abbreviations used are: T2D, type 2 diabetes; miRNA, microRNA; DNM, dynamin; Chr, chromosome; LP, low protein; G, glucose; 2DG, 2-deoxy-D-glucose; HBSS, Hanks' balanced salt solution; cDNA, complementary DNA; ANOVA, analysis of variance; TX, thymidine kinase promoter.

trolled by their own promoters. PrimiR-199a/b gene sequences are highly conserved between species and share the same seed sequence, potentially targeting the same genes. Consequently, the three primiRNAs of the miR199 family are able to generate both mature miRNAs: 199-3p and 199-5p (15). primiRNA gene expression is regulated by different mechanisms. For instance, although primiR-199a2 is regulated by transcription factors such as TWIST1 and EGR1 on Chr1, methylation status regulates both primiR-199a1 and primiR-a2 promoters on both Chr1 and Chr19 (14, 16, 17). The mature forms have different targets and, consequently, different biological effects. Mature miR-199-3p controls tumor proliferation and invasion by targeting mRNA of key proteins such as mTOR (18–20). miR-199-5p regulates hypoxia-inducible factor 1a (HIF-1a) in cardiomyocytes (21). Mitogen-activated protein kinases and extracellular signal-regulated kinase are also under the influence of miR-199 (17, 22).

miRNAs are critical regulators of insulin secretion and pancreas development as well as glucose homeostasis by modulating β-cell function and mass expansion in adult mice (23–28). Remarkably, several miRNAs are known to be altered in obesity and diabetes (29–33). Based on these and other observations, it is believed that β-cell function, turnover, and differentiation are preserved in part by a delicate balance between positive and negative regulatory miRNAs (31). We reported previously that defective insulin secretion is associated with alterations in a subset of miRNAs in the offspring of dams exposed to low protein (LP) during pregnancy (fetal programming) (34). In these studies, we showed that protein restriction during pregnancy induces decreased expression of mTOR protein and permanent up-regulation of islet miR-199-3p expression in islets of LP-exposed offspring (34). Anti-miR-199-3p treatment rescued mTOR protein levels and insulin secretion in islets from LP mice, suggesting that LP induces changes in mTOR levels and insulin secretion in part by regulating miR-199 (34). In addition to these findings, the role of miR-199 has been studied in pancreatic islets and β-cell lines. miR-199-3p and miR-199-5p are overexpressed in diabetic db/db mice and nonobese diabetic Goto-Kakizaki (GK) rats (30, 31). Remarkably, up-regulation of miR-199-5p led to an insulin secretion defect in mouse insulinoma cells (MIN6) and mouse islets and diminished the insulin content in islets, whereas miR-199-3p increased the number of apoptotic cells in MIN6 cells, rat islets, and dissociated human islet cells (31). Furthermore, miR-199-5p has been implicated in β-cell loss (35). Islet miRNAs can contribute to the increased concentrations of miR-199 in plasma from patients with type 2 diabetes (6). miR-199 in plasma contributes to insulin resistance by targeting insulin-sensitive glucose transporter 4 (GLUT4) in peripheral tissues like skeletal muscle (36). Among all miRNAs that target GLUT4, only miR-199 is up-regulated in diabetic patients, suggesting that miR-199 could be a novel biomarker for T2D patients (36). Although these findings support evidence of a role of miR-199 in islet function and diabetes, very little is known about how miR-199 expression is regulated.

In this study, we explore the mechanisms that regulate miR-199 in β-cells as a model to understand transcriptional regulation of miRNAs. This work demonstrated that the miR-199 family is acutely regulated by glucose levels in pancreatic mouse islets and MIN6 cells at the level of primiRNA expression, leading to modifications of mature miR-199 levels. We report that membrane depolarization–induced calcium release controls the activity of primiR-199a2 promoter activity in β-cells. Calcium influx also mediates mature miR-199 expression in mouse islets. Our findings add new insights into the acute regulation of miR-199 in pancreatic islets, a miRNA with major negative impact in β-cell function and whose expression is induced in pathological states.

Results

The miR-199 family and its precursors are expressed equally in female and male islets

The three miR199 precursors (the primiR-199a1, primiR-199a2, and primiR-199b genes) are able to generate two mature miRNAs: miR-199-3p and miR-199-5p (Fig. 1*A*). Therefore, we first assessed the expression levels of primiR-199 genes in mouse pancreatic islets. We used the highly abundant miR-7 family as a positive control. There was no difference between female and male islets in expression of primiR-199a1, primiR-199a2, primiR-199b, primiR-7a1, and primiR-7a2 (Fig. 1*B*). Interestingly, primiR-199a1 is the most-expressed in mouse islets, 30% higher than primiR-199a2 (Fig. 1*C*). In contrast, primiR-199b exhibits very low expression in islets (Fig. 1*C*). primiR-7a2 is 10-fold more expressed than primiR-199a1 and primiR-199a2 (Fig. 1*C*). The same pattern of expression was observed in MIN6 insulinoma cells: primiR-7a2 > primiR-7a1 > primiR-199a1 > primiR-199a2 > primiR-199b (Fig. 1*D*). Next we determined expression of the mature forms of both miR-199s. Males and females expressed the same levels of miR-199-3 and miR-199-5p (data not shown). Furthermore, within female islets (Fig. 1*E*) and male islets (Fig. 1*F*), expression of miR199-3p and 199-5p was similar, validating the primiRNA results.

primiR-199 and miR-199 expression is induced by glucose in mouse islets

To determine whether glucose regulates the expression of miR-199, we assessed the RNA expression of primiR-199a1 and primiR-199a2 in mouse islets exposed to 5.5 mM or 10 mM glucose (G) for 24 h (Fig. 2). primiR-199a1 (1.6-fold) and primiR-199a2 (2.3-fold) expression was enhanced in islets cultured in 10 mM G compared with 5.5 mM G, respectively (Fig. 2*A*). It is expected that increased levels of primiRNA genes are accompanied by an elevation of mature miR-199-3p and miR-199-5p levels (Fig. 1*A*). miR-199-3p was 1.5-fold increased, whereas a nonsignificant trend of increase in mature miR-199-5p (1.4-fold, $p = 0.08$) was observed in islets treated with 10 mM G (Fig. 2*B*) after 24 h. Previous data indicate that glucose-induced miR-199-3 and miR-199-5p expression in rodent islets depends on the magnitude and time of exposure to hyperglycemia (30, 31). Therefore, we assessed mature miR-199 expression in mouse islets after 48 h in 5.5 mM, 11 mM, and 16.5 mM G (Fig. 3). Prolonging the treatment to 48 h resulted in up-regulation of miR-199-3p expression by 1.9-fold at 11 mM G and 2-fold at 16 mM G (Fig. 3*A*). Although a trend of increase of miR-199-5p expression was observed (1.9-fold) at 11 mM G, mouse islets in

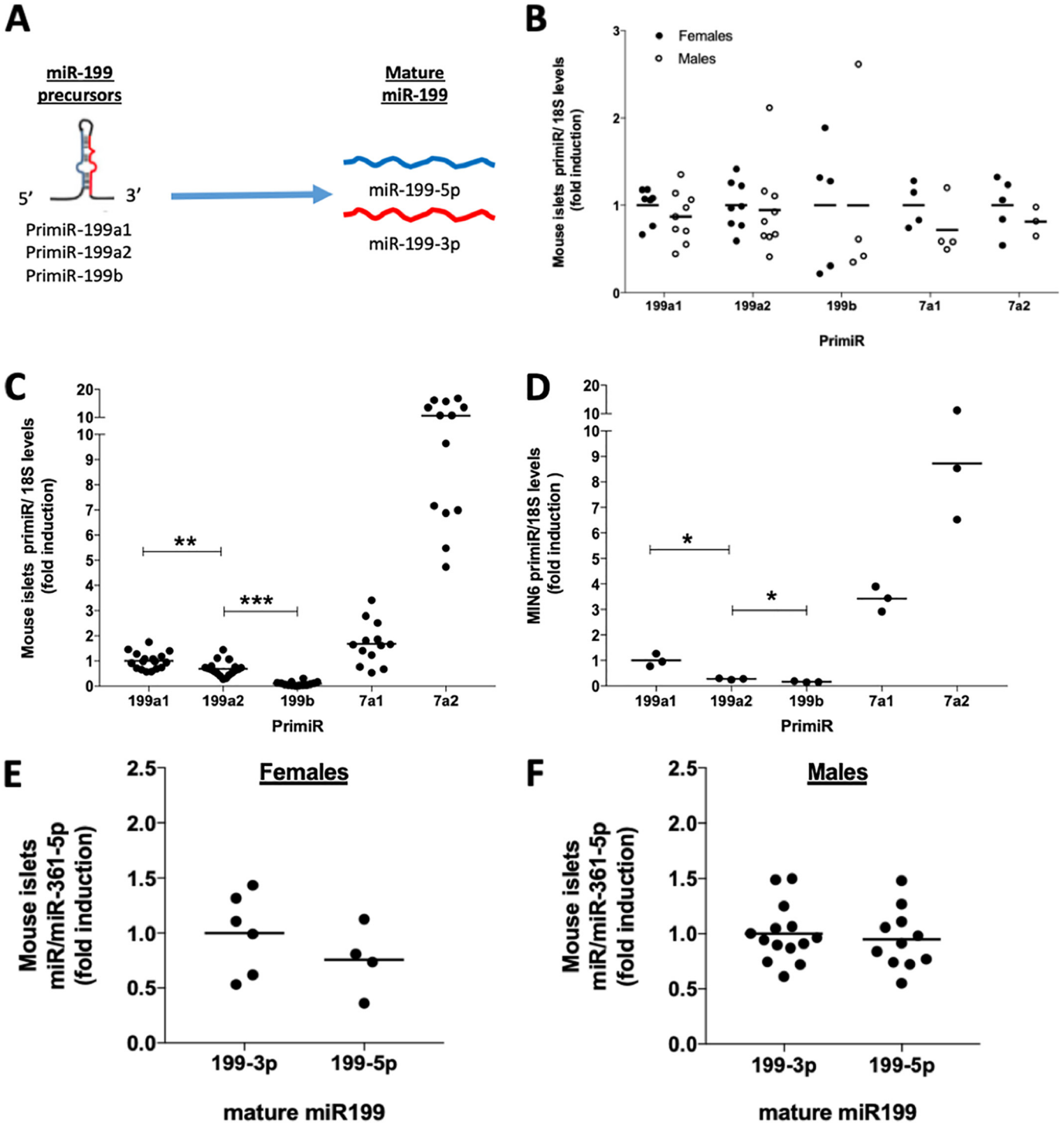

Figure 1. miR-199 family expression in mouse islets and MIN6 cells. *A*, the miR-199 family is comprised of the precursor primiR-199a1, primiR-199a2, and primiR-199b genes, encoded within introns of DNM genes in the opposite orientation. The primiRNA genes are transcribed by RNA polymerase II, processed in the nucleus into its precursor (premiRNA) by Drosha, and then into mature miRNA by Dicer. All primiRNAs generate the mature miR-199-3p and miR-199-5p. The mature miRNA targets different mRNA to regulate cellular function. *B*, primiRNA expression in female and male mouse islets. Data are shown as -fold induction to female expression. *C* and *D*, primiRNA expression in mouse islets (*C*) and MIN6 cells (*D*). Data are presented as -fold induction to primiR-199a1 expression. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; determined by one-way ANOVA followed by Tukey's post hoc test against every other group within the miR-199 family. primiR-7a1 and primiR-7a2 are plotted as positive controls for primiRNA expression in islets. 18S mRNA levels were used as internal controls. *E* and *F*, mature miR-199 expression in female islets (*E*) and male islets (*F*). Results are expressed as -fold induction to miR-199-3p. miR-361–5p was used as an internal control.

16.6 mM G for 48 h showed a 2.7-fold increase in miR-199-5p expression (Fig. 3*B*).

We also tested glucose effects on primiRNA expression in MIN6 cells (Fig. 3). Because MIN6 cells are normally cultured in DMEM containing 25 mM G (37–39), we assessed primiRNA expression in MIN6 cells cultured in 5 mM G *versus* 25 mM G, as described previously for gene profiling (39). After 3 h, the expression of primiR-199a1, primiR-199a2, and primiR-199b

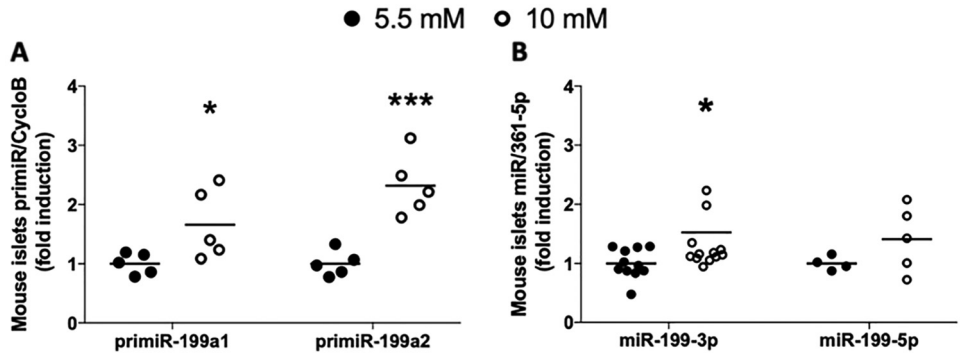

Figure 2. Glucose levels affect primiR-199 and miR-199 expression in mouse pancreatic islets. *A*, primiR-199a1 and primiR-199a2 expression in mouse islets after 24 h in 5.5 mM or 10 mM glucose. *B*, the same as in *A*, except that miR-199-3p and miR-199-5 were assessed. *, $p < 0.05$; ***, $p < 0.001$; determined by *t* test compared with 5.5 mM glucose. CycloB mRNA levels were used as internal controls for primiRNA expression, whereas miR-361-5p was the control for miRNA expression. Data are expressed by -fold induction to 5.5 mM glucose.

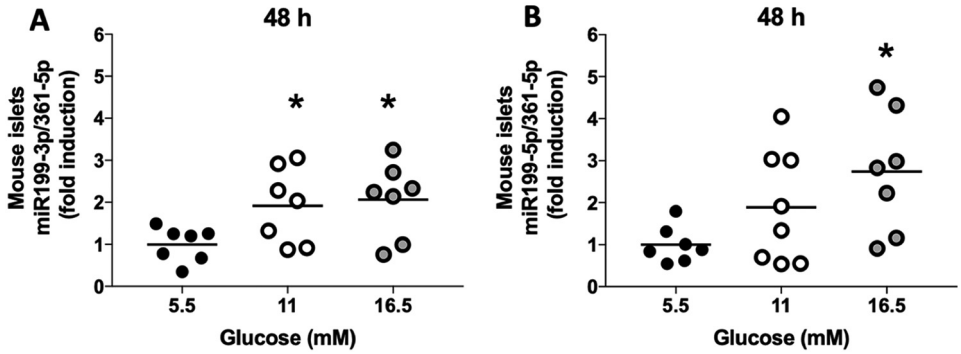

Figure 3. Prolonged hyperglycemia increases mature miRNA expression. *A*, miR-199-3p expression in mouse islets after 48 h in in 5. 5 mM, 11 mM, or 16.5 mM glucose. *B*, the same as in *A*, except that miR-199-5p levels were assessed. *, $p < 0.05$; determined by one-way ANOVA followed by Dunnett's post hoc test compared with 5.5 mM glucose. miR-361–5p was the control for miRNA expression. Data are expressed by -fold induction to 5.5 mM glucose.

was, respectively, ~60%, 40%, and 55% higher in 25 mM G compared with cells kept in 5 mM G (Fig. 4, *A–C*). Exposure of MIN6 cells to 25 mM G for 24 h further increased primiR-199a1, primiR-199a2, and primiR-199b expression compared with 5 mM G (Fig. 4, *D–F*). Although MIN6 cells are normally cultured in 25 mM glucose, we tested whether lower-glucose stimulation with 10 mM for 24 h would also induce primiR-199a2 expression and found higher mRNA levels for primiR-199a2 compared with the 2 mM glucose condition (Fig. S1*A*). No difference in Ins2 or CycloA expression was observed (Fig. S1, *B* and *C*).

Membrane depolarization increases primiR-199a2 expression in β-cells

The finding that glucose levels control primiRNA and miRNA expression in islets and MIN6 cells led us to investigate the mechanisms of glucose induction of primiRNA expression. We first assessed whether membrane depolarization would rescue primiRNA expression in MIN6 cells. To address this, MIN6 cells were cultured in 5 mM G with or without 30 mM KCl for 3 or 6 h (Fig. 5). Again, when cultured in 5 mM G, the expression of primiR-199a1, primiR-199a2, and primiR-199b was lower than with 25 mM G at 3 (Fig. 5, *A–C*) and 6 h (Fig. 5, *D* and *E*). KCl-induced depolarization increased primiR-199a2 mRNA expression at 3 h (Fig. 5*B*), and prolonging KCl exposure to 6 h elevated primiR-199a2 mRNA to levels above 25 mM G (Fig. 5*E*). The KCl effect appeared to be specific to primiR-199a2 because mRNA expression of primiR-199a1 and primiR-199b was not sensitive to KCl at 3 (Fig. 5, *A* and *C*) or 6 h (Fig. 5, *D* and *F*). Importantly, β-cell–related genes such as *ins2* and *pdx-1* did not change after 3 or 6 h in 5 mM G with or without KCl (Fig. S2).

primiR-199a2 promoter activity is regulated by glucose metabolism but not by growth factors

We then focused on regulation of primiR-199a2 RNA by glucose. To investigate whether primiR-199a2 RNA expression was regulated at the transcriptional level, we assessed primiR-199a2 promoter activity by transiently transfecting MIN6 cells with a construct containing the putative 1349-bp primiR-199a2 promoter sequence coupled to firefly luciferase (16). After 3 h, primiR-199a2 promoter activity was about 30% higher in 25 mM G compared with 5 mM G (Fig. 6*A*). Growth factor starvation in MIN6 cells cultured in serum-free medium did not affect primiR-199a2 promoter activity, suggesting that the transcriptional activity of the primiR-199a2 promoter is specific to glucose and not to growth factors/hormones (Fig. 6*A* and Fig. S3). Inhibition of glycolysis by coculturing MIN6 cells with different concentrations of 2-deoxy-D-glucose (2DG) and glucose showed a dose response in primiR-199a2 promoter activity to different concentrations of 2DG (Fig. 6*B*).

β-cell membrane depolarization and calcium influx through L-type channels induce primiR-199a2 promoter activity

The previous studies suggest that glycolysis is involved in regulation of primiR-199a2 transcription. The next step was to determine whether calcium influx by membrane depolarization

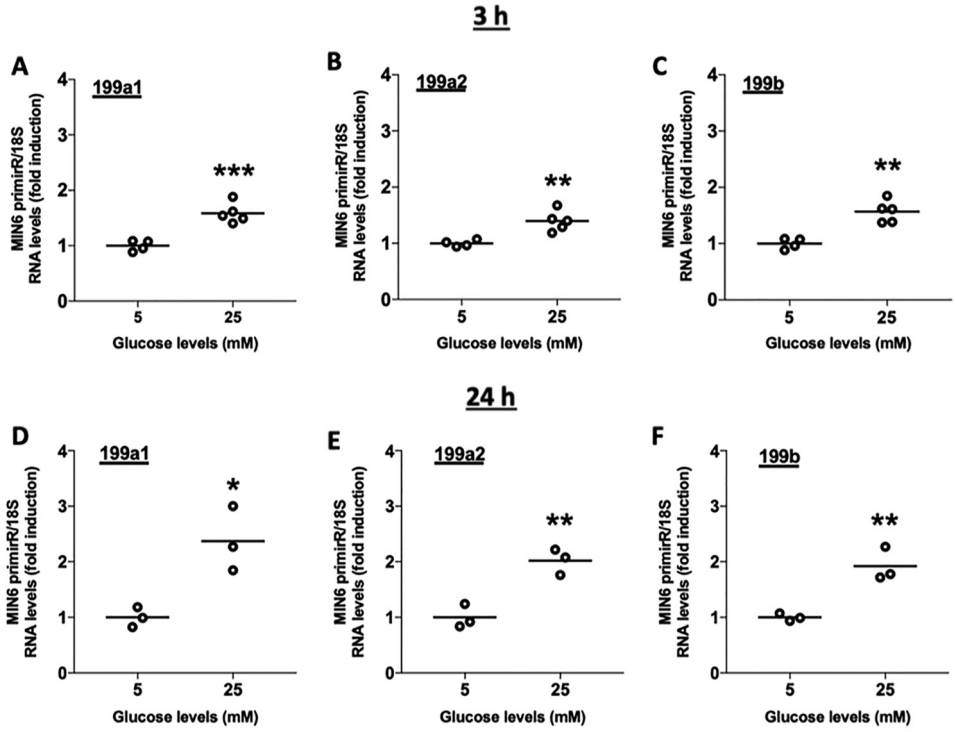

Figure 4. primiR-199 expression is regulated by glucose in MIN6 cells. *A–C*, primiRNA expression in MIN6 cells after 3 h in 5 mM or 25 mM glucose. *D–F*, the same as in *A–C*, except that MIN6 cells were cultured for 6 h. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; compared with 5 mM glucose determined by *t* test. 18S mRNA levels were used as internal controls for primiRNA expression. Data are presented as -fold induction to 5 mM.

would regulate primiR-199a2 promoter activity. We cultured MIN6 cells in either 5 or 25 mM G with or without 30 mM KCl for 3 and 6 h. This dose was chosen from the dose–response curve of primiR-199a2 promoter activity to KCl when MIN6 cells were cultured in 5 mM G (Fig. S4). Again, promoter activity was greater in 25 mM G at 3 (~50%) and 6 h (~20%) than in 5 mM G (Fig. 7, *A* and *B*). Interestingly, KCl has no effect on primiR-199a2 when cells were cultured in 25 mM G, suggesting maximum promoter activity. In 5 mM G, KCl treatment mildly increased primiR-199a2 promoter activity at 3 h (Fig. 7*B*), but transcriptional activity similar to 25 mM G was observed after 6 h (Fig. 7*C*). These results are reminiscent of the findings observed in primiRNA expression studies (Fig. 5). In addition, β-cell membrane depolarization induced by pharmacological inhibition of the potassium channel with tolbutamide (200 μM) also induced primiR-199a2 promoter activity (Fig. S5). To determine the role of calcium influx in transcriptional regulation of primiR-199a2, MIN6 cells transfected with the primiR-199a2 promoter were treated with different concentrations of the L-type calcium channel blocker nifedipine. PrimiR-199a2 promoter activity was reduced by 10%–15% after culture in nifedipine at the 10–50 μM range (Fig. 8*A*). A major drop in primiR-199a2 promoter activity was observed at 100 and 200 μM (Fig. 8*A*). The results obtained with the calcium channel blocker prompted us to test whether calcium influx also regulates mature miR199 levels in mouse islets. We cultured isolated islets for 48 h in 5.5 mM G or 16 mM with or without diazoxide. Remarkably, diazoxide blunted glucose-induced upregulation of miR-199-3p by high glucose (Fig. 8*B*). These findings indicate that glucose-induced calcium influx by β-cell membrane depolarization regulates miR-199 expression by stimulating primiR-199a2 gene transcription.

Discussion

Although T2D pathogenesis is multifactorial, growing evidence supports a role of miRNAs in regulation of glucose homeostasis and metabolism (5, 26, 27, 34, 40, 41). miRNAs are important candidates, as a single miRNA can regulate multiple target genes that ultimately result in widespread changes in the protein levels of hundreds to thousands of genes. Importantly, little is known about the molecular mechanisms that govern miRNA expression, and unraveling the mechanisms could therefore lead to development of new strategies to prevent or treat diabetes. Several miRNAs have been implicated in β-cell development, survival, and function (23–28). Here we report that glucose acutely induces miR-199 expression in pancreatic mouse islets and MIN6 insulinoma cells. These findings are important, as we and others have found miR-199 is increased in pathological states such as obesity, diabetes, and fetal exposure to low protein (30, 31, 34). miR-199 has also been shown to negatively impact β-cell function and regulate the levels of the nutrient sensor mTOR (31, 34). In addition, this study demonstrates that primiR-199a1, primiR-199a2, and primiR-199b RNA levels are regulated by glucose in islets and MIN6 cells and that induction of primiR-199 in mouse islets leads to an increase in the mature forms of miR199 (miR-199-3p and miR-199-5p; Figs. 2 and 3). Finally, we identify that, among the miR-199 family, primiR-199a2 was the only primiRNA regulated by glucose-induced depolarization, and further studies using primiR-199a2 promoter–reporter assays support the concept that

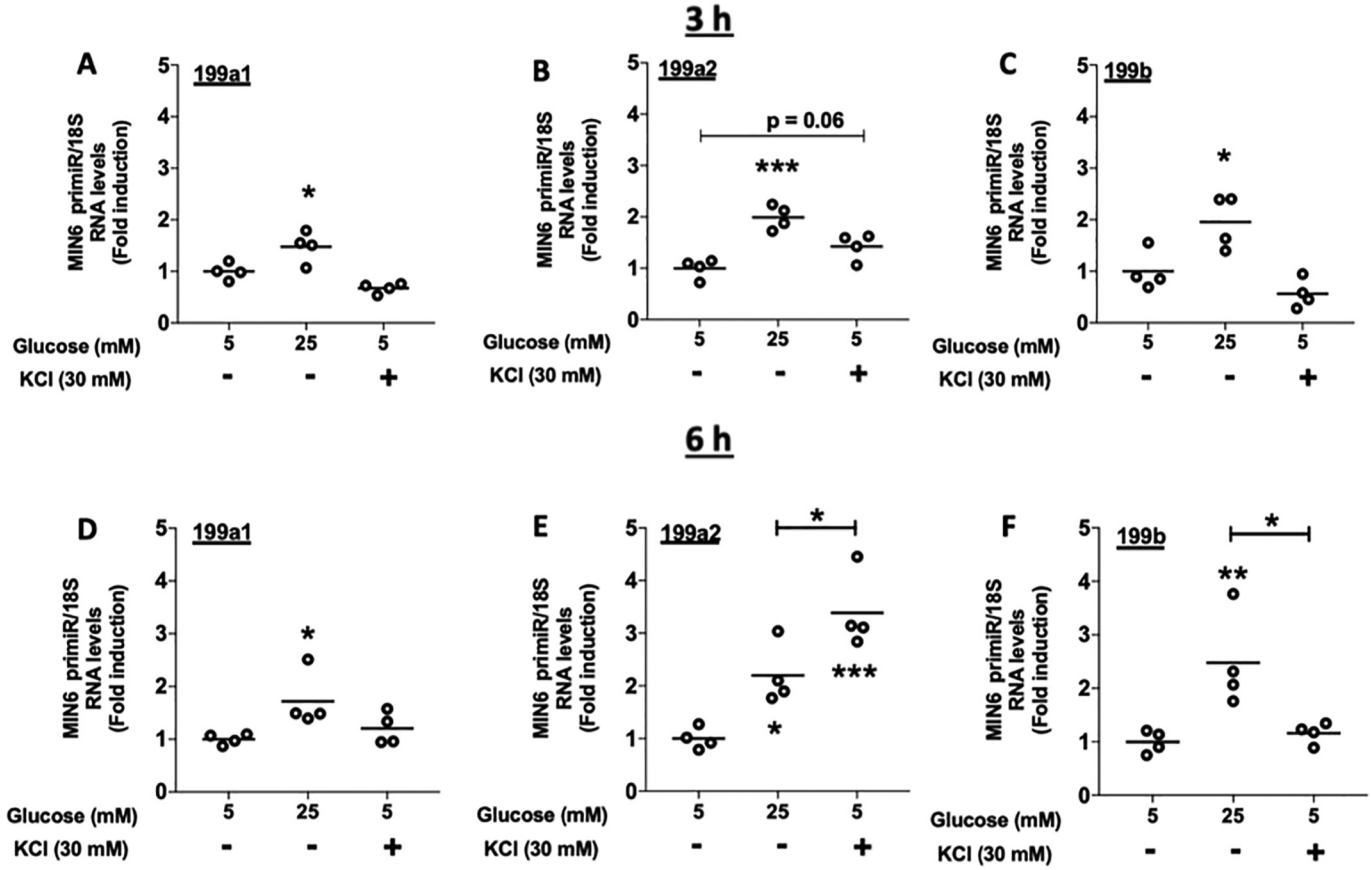

Figure 5. KCL-induced membrane depolarization increases primiR-199a2 expression in MIN6 cells. *A–C*, primiR-199a, primiR-199a2, and primiR-199b mRNA levels, respectively, in MIN6 cells under 25 mM glucose or 5 mM glucose with or without 30 mM KCl for 3 h. *D–F*, the same as in *A–C*, except that MIN6 cells were treated for 6 h. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; compared with 5 mM glucose or the indicated groups by the *line* and determined by one-way ANOVA followed by Tukey's post hoc test against every other group. 18S mRNA levels were used as internal controls for primiRNA expression. Data are shown as -fold induction to 5 mM glucose without KCl.

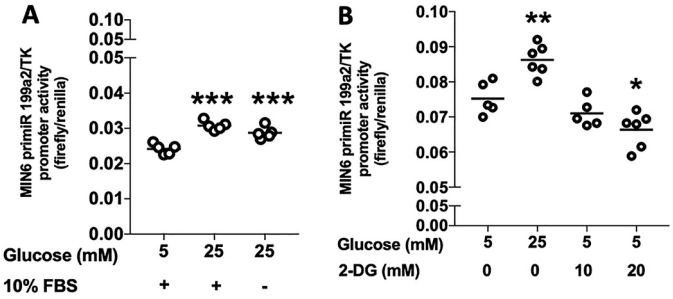

Figure 6. Glucose but not serum deprivation regulates primiR-199a2 promoter activity in MIN6 cells. *A*, primiR-199a2 promoter activity measured by firefly luciferase activity in MIN6 cells transiently transfected overnight with Lipofectamine 3000 and cultured in 5 mM glucose or 25 mM glucose with or without 10% FBS. *B*, the same as in *A*, except that transfected MIN6 cells were starved with different concentration of 2DG in the presence of FBS. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; determined by one-way ANOVA followed by Dunnett's post hoc test compared with 5 mM glucose in *A* or 5 mM glucose and 0 mM 2DG in *B*. *Renilla* luciferase activity was used as a transfection efficiency control.

glucose metabolism, depolarization, and calcium regulate the transcription of primiR-199a2 (Figs. 4-8).

Expression studies showed no gender differences in islet expression of primiR-199, and primiR-199a1 was the most abundantly expressed primiRNA, followed by primiR-199a2 and primiR-199b in mouse islets and MIN6 cells (Fig. 1, *C* and *D*). We found that glucose regulated the expression of all primiRs-199 in islets, and these results were also validated in MIN6 cells, a transformed β-cell line. It is intriguing but not surprising that the most expressed primiR-199 isoforms in islets, primiR-199a1 and primiR-199a2, are regulated by different mechanisms in β-cells. Both primiRNAs' expression is lower with low glucose levels in MIN6 cells. However, KCl-induced depolarization only increases the expression of primiR-199a2, suggesting that transcription of this primiRNA is sensitive to depolarization and calcium influx. This also suggests that glucose regulates primiR-199a1 by mechanisms that are likely independent of calcium influx. Studies using a primiR-199a2 promoter-luciferase construct further demonstrated that the regulation of primiR-199a2 occurs at the transcriptional level and appears to be specific to glucose and not growth factors. Studies with 2DG suggested that glucose metabolism is necessary for the induction of primiR-199a2 transcription by glucose. The induction of primiR-199a2 transcriptional activity by tolbutamide and KCl further implicated K_{ATP} channels, depolarization, and induction of calcium influx as mechanisms responsible for primiR-199a2 transcription induced by glucose. This suggests that primiRNA expression in β-cells shares the pathways described for glucose-regulated genes in β-cells and point to a role of environmental glucose in regulation of miRNA transcription. This work also suggests that glucose can modulate gene expression by regulation of miRNAs.

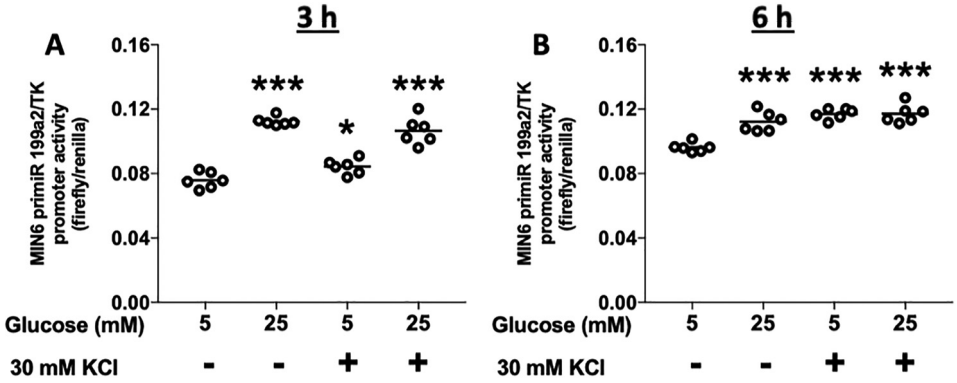

Figure 7. Effects of KCL on primiR-199a2 promoter activity in MIN6 cells. *A*, primiR-199a2 promoter activity measured by firefly luciferase activity in MIN6 cells transiently transfected overnight with Lipofectamine 3000 and cultured in 5 or 25 mM glucose with or without 30 mM KCl for 3 h. *B*, the same as in *A*, except that cells were treated for 6 h. *, $p < 0.05$; ***, $p < 0.001$; compared with 5 mM glucose without KCl and determined by one-way ANOVA followed by Tukey's post hoc test. TK *Renilla* luciferase activity was used as a transfection efficiency control.

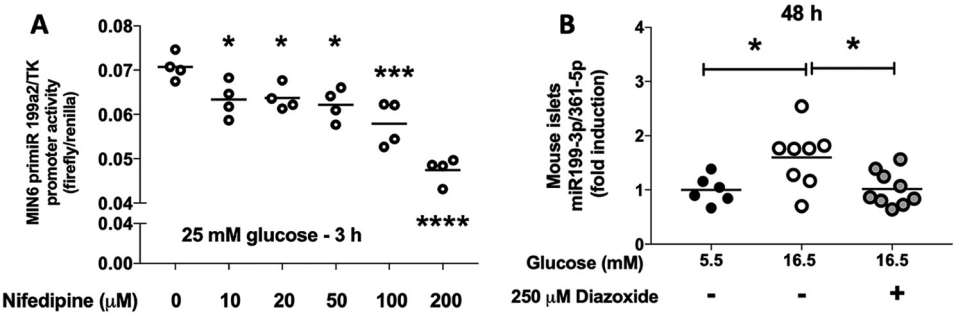

Figure 8. Calcium influx induces primiR-199a2 promoter activity and mature miRNA expression. *A*, primiR-199a2 promoter activity measured by firefly luciferase activity in MIN6 cells transiently transfected overnight with Lipofectamine 3000 and cultured in 25 mM glucose for 3 h with increasing concentrations of the calcium channel blocker nifedipine (0–200 μM). *, $p < 0.05$; ***, $p < 0.001$; determined by one-way ANOVA followed by Dunnett's post hoc test against the control group (0 μM nifedipine). TK *Renilla* luciferase activity was used as a transfection efficiency control. *B*, miR-199-3p expression in mouse islets after 48 h in in 5.5 mM or 16.5 mM glucose with or without 250 μM diazoxide. *, $p < 0.05$; determined by one-way ANOVA followed by Dunnett's post hoc test compared with 5.5 mM glucose. miR-361–5p was the control for miRNA expression. Data are expressed by -fold induction to 5.5 mM glucose.

How calcium influx ultimately results in primiRNA transcription in β-cells is unclear, but it is possible that activation of multiple calcium-regulated cell signaling pathways, including calmodulin kinases, PKA, and PKC, among others, could be involved. Although the primiR-199a1 promoter is not well defined and its regulation relies mostly on methylation, the primiR-199a2 promoter contains putative binding sites for Twist-1 and early growth response 1 (EGR1) among other transcription factors (14, 16). These transcription factors have been shown to control primiR-199a2 expression in the mouse neural crest–derived cell line (N2a) and in the mouse embryonic fibroblast cell line NIH-3T3 by binding to the promoter (14, 16). EGR1 is an early response gene that is rapidly induced in MIN6 cells and islets by glucose, KCl, and tolbutamide in a calcium-dependent manner (37, 42). EGR1 has also been implicated in regulation of pdx1 and insulin gene expression and β-cell function and survival (43–45). Glucose-regulated EGR1 induction by calcium has been shown to be mediated by calmodulin kinases, PKA, and calcium activation of the serum response factor (37). It is conceivable that glucose can modulate different biological processes in β-cells by a cascade of events that include expression of early response genes such as EGR1 and that this transcription factor can ultimately induce miRNA expression to amplify the glucose response by modulation of multiple genes that could regulate multiple biological processes. Further studies need to be designed to determine whether EGR1 or other transcription factors are involved in primiR-199a2 expression in β-cells.

Our results confirm previous work in rodent models showing that the miR-199 family consists of glucose-sensitive miRNAs. Nesca *et al.* (31) assessed miRNA expression in a model of severe hyperglycemic (diabetic db/db mice) or mild hyperglycemia after administration of a high-fat diet. They reported that severely hyperglycemic db/db mice (5 months old) exhibit increases in miRNA expression, including miR-21 (5-fold), miR-132 (5-fold), miR-199-5p (4.5-fold), and miR-199-3p (8-fold). In contrast, the magnitude of miRNA induction in islets from mice with mild hyperglycemia was significantly lower, suggesting that the magnitude of hyperglycemia plays a role. In a separate study, miRNA expression was assessed in islets from hyperglycemic GK rats and in *in vitro* culture of WT islets for 24 h at different glucose concentrations (2.8 mM, 8.3 mM, and 16.8 mM glucose) (30). The results of these studies showed that 24 miRNAs were up-regulated (including miR-199-3p and miR-199-5p) in GK diabetic rat islets. In contrast, only two miRNAs with -fold changes as high as 1.5 were up-regulated in islets cultured in high glucose. Taken together, these studies suggest that the magnitude of hyperglycemia in *in vivo* models differently affects the expression of miRNAs and that the magnitude of glucose induction of miRNA expression is lower in short-term *in vitro* culture with high glucose. Here we show that prolonging glucose exposure *in vitro* from 24 to 48 h

induces greater mature miR-199-3p expression. However, we did not observe any difference between 11 mM G and 16.5 mM G after 48 h. In contrast, miR-199-5p up-regulation by glucose only reached statistical significance after 48 h of exposure to 16.5 mM G. These findings advance our current knowledge by providing insights into the mechanisms responsible for glucose induction of miR-199 expression in β-cells.

We conclude that miR-199 is a glucose-sensitive miRNA at the primiRNA level in mouse pancreatic islets and mouse insulinoma cells. Although all primiR-199s are higher under hyperglycemia conditions, only primiR-199a2 is regulated by glucose metabolism, cell depolarization, and calcium influx in β-cells. Importantly, calcium also up-regulates mature miR-199 expression in mouse islets. These data validate published data showing higher miR-199 levels in chronic models of hyperglycemia in diabetes and suggest that glucose induction of miR-199 expression could ultimately alter biological processes in models of chronic hyperglycemia (30, 31, 34, 36). This information adds to the already known repertoire of effects of miR-199 in metabolic diseases. Importantly, several lines of evidence support a role of this miRNA as a candidate in the pathogenesis of T2D: miR-199 is increased in islets from obese and diabetic mice and rats and negatively impacts β-cell function (30, 31); it is induced in islets of adult offspring of dams fed a low-protein diet during pregnancy (β-cell programming) (34); it suppresses mTORC1 expression, a kinase implicated in β-cell survival, proliferation, and function (47, 48); diabetic patients exhibit greater miR-199 levels in the blood (36); and miR-199 inhibits GLUT4 expression (36).

Experimental procedures

Pancreatic islet isolation

All procedures were approved by University of Miami institutional animal care and use committee (institutional animal care and use committee protocol 18-168). Islets were isolated by collagenase digestion (49). 3–4 ml of 1 mg/ml collagenase (Collagenase P, Sigma-Aldrich) in Hanks' balanced salt solution (HBSS; Corning Cellgro) was injected into the common bile duct to inflate the pancreas using a 5-ml syringe and 30-gauge needle. Then the pancreas was carefully removed, inserted into a 15-ml conical tube with 1 ml collagenase, and incubated at 37 °C for 16 min using a temperature-controlled water bath with mild shaking. After digestion, the tubes were shaken vigorously to break down the tissue. To stop tissue digestion, 10 ml of cold HBSS supplemented with 10% fetal bovine serum was added to the tubes on ice. Then the digested pancreas was centrifuged for 2 min at 200 × *g*. The supernatant was removed, and the pancreas washed twice with 10 ml of 10% FBS in HBSS. After washing, digested tissue was filtered through a stainless steel tea ball strainer to remove undigested pieces of pancreas. The digested tissue was resuspended in 5 ml of Histopaque solution (Histopaque-1077, Sigma-Aldrich), and 5 ml of plain HBSS was carefully added on top. Tubes were centrifuged for 20 min at 10 °C and 966 × *g* with minimum acceleration and deceleration. Islets retained at the Histopaque–HBSS interface were collected in a new 15-ml tube, and HBSS was added up to 15 ml. Islets were centrifuged at 966 × *g* for 4 min. After discarding the supernatant, islets were resuspended and plated in nontreated plastic Petri dishes (VWR) containing RPMI 1640 (Corning Cellgro) supplemented with 10% FBS, 1% penicillin and streptomycin, and 5.5 mM glucose. After overnight culture in 5.5 mM glucose, islets were handpicked for studies. Islets were cultured at different glucose levels, as indicated in the figure legends, and used for primiRNA or miRNA expression. The potassium channel activator diazoxide (250 μM) was used to inhibit calcium influx in isolated mouse islets.

Mouse insulinoma cells culture

MIN6 cells were maintained in DMEM (high glucose, 25 mM) supplemented with 10% FBS, 1 mM sodium pyruvate, 4 mM L-glutamine, and 1% penicillin and streptomycin as described previously (37). Cells were used for experiments at 50%–70% confluence. MIN6 cells were tested for glucose-stimulated insulin secretion before experiments (Fig. S6, *A* and *B*). For this, MIN6 cells were preincubated in 2 mM glucose KRB buffer (125 mM NaCl, 5.9 mM KCl, 2.56 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM Hepes, and 1 mg/ml BSA) for 2 h. Then cells were incubated with 2 mM glucose KRB buffer for 1 h, followed by 1 h in 25 mM glucose. Insulin was determined by ultrasensitive mouse ELISA kit (Alpco) according to the company's recommendations. For primiRNA expression, MIN6 cells were cultured in 5 or 25 mM glucose as described previously for gene expression (39, 46).

Quantitative real-time PCR

Total RNA containing large and small RNAs was isolated using the mirVana microRNA isolation kit (Invitrogen). To assess primiRNA and mRNA levels, cDNA was synthesized using 1 μg of total RNA using random hexamers and reverse-transcribed using Superscript II (high-capacity cDNA reverse transcription kit, Applied Biosystems). SYBR Green (Quantabio) or TaqMan (Quantabio) Master Mix was used to amplify specific genes and primiRNAs. To assess mature miRNA expression, we used the TaqMan Advanced microRNA cDNA Synthesis Kit (Applied Biosystems) to synthesize miRNA cDNA from 10 ng of total RNA. TaqMan Master Mix was used to quantify mature miRNA expression. All primiRNA and miRNA assays (primers) were from Thermo Fisher Scientific: primiR-199a1 (Mm03306505_pri), primiR-199a2 (Mm03307203_pri), primiR-199b (Mn03307205_pri), primiR-7a1 (Mm03307287_pri), primiR-7a2 (Mn03307288), miR-199-3p (Mmu480983_mir), miR-199-5p (Rno480984_mir), miR-7a1–3p (Mmu482999_mir), miR-7a2–3p (Mmu481267_mir), and miR-7b-3p (Mmu482329_mir). Cyclo A or B or 18S (Mn03928990) was used as an internal control for mRNA and primiRNA expression, whereas miRNA expression was normalized by miR-361-5p (Mmu481127_mir). Real-time PCR was performed on an ABI 7000 (Applied Biosystems).

Luciferase reporter assay

MIN6 cells at 60%–70% confluence in 24-well plates were transfected using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's recommendations. The putative 1349-bp promoter for primiR-199a2 was cloned to the pGL4-1.2 reporter coupled to firefly luciferase, kindly provided by Dr. Hilfiker-Kleiner. The cells were transfected overnight with 500 ng of miR-199a2 construct (16) and recovered in high-

glucose DMEM before experiments. Then MIN6 cells were cultured in low (5 mM) or high (25 mM) glucose with or without KCl (Sigma-Aldrich), 2DG (Sigma-Aldrich), nifedipine (Sigma-Aldrich), and tolbutamide (Sigma-Aldrich). The thymidine kinase promoter-*Renilla* (100 ng) was cotransfected and used as a transfection efficiency control. All luciferase results are shown as firefly/*Renilla* activity and were obtained by the Dual-Luciferase® Reporter Assay System (Promega).

Statistics

Data are represented as mean ± S.E. Student's *t* test was employed to assess statistically significant differences between means of two groups, *e.g.* miRNA and primiRNA expression in males *versus* females (Fig. 1, *B*, *E*, and *F*), miR199-3p *versus* miR199-5p (Fig. 2), and low *versus* high glucose (Fig. 4). One-way analysis of variance followed by Tukey's post hoc test was used to identify differences between three or more groups in MIN6 cells and mouse islets (Figs. 1, *C* and *D*, 5, 7, and 8*B*). One-way ANOVA followed by Dunnett's post hoc test was performed to compare two or more groups (conditions) with the control group (Figs. 3, 6, and 8*A*). Results were considered statistically significant when the *p* value was less than 0.05.

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References

1. Alejandro, E. U., Gregg, B., Blandino-Rosano, M., Cras-Méneur, C., and Bernal-Mizrachi, E. (2015) Natural history of beta-cell adaptation and failure in type 2 diabetes. *Mol. Aspects Med.* **42,** 19–41 CrossRef Medline
2. Lango, H., UK Type 2 Diabetes Genetics Consortium, Palmer, C. N., Morris, A. D., Zeggini, E., Hattersley, A. T., McCarthy, M. I., Frayling, T. M., and Weedon, M. N. (2008) Assessing the combined impact of 18 common genetic variants of modest effect sizes on type 2 diabetes risk. *Diabetes* **57,** 3129–3135 CrossRef Medline
3. Morris, A. P., Voight, B. F., Teslovich, T. M., Ferreira, T., Segre, A. V., Steinthorsdottir, V., Strawbridge, R. J., Khan, H., Grallert, H., Mahajan, A., Prokopenko, I., Kang, H. M., Dina, C., Esko, T., Fraser, R. M., *et al.* (2012) Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes. *Nat. Genet.* **44,** 981–990 CrossRef Medline
4. Sandovici, I., Hammerle, C. M., Ozanne, S. E., and Constância, M. (2013) Developmental and environmental epigenetic programming of the endocrine pancreas: consequences for type 2 diabetes. *Cell Mol. Life Sci.* **70,** 1575–1595 CrossRef Medline
5. Filios, S. R., and Shalev, A. (2015) β-Cell microRNAs: small but powerful. *Diabetes* **64,** 3631–3644 CrossRef Medline
6. Zhang, A., Li, D., Liu, Y., Li, J., Zhang, Y., and Zhang, C. Y. (2018) Islet β cell: an endocrine cell secreting miRNAs. *Biochem. Biophys. Res. Commun.* **495,** 1648–1654 CrossRef Medline
7. Xie, X., Lu, J., Kulbokas, E. J., Golub, T. R., Mootha, V., Lindblad-Toh, K., Lander, E. S., and Kellis, M. (2005) Systematic discovery of regulatory motifs in human promoters and 3′ UTRs by comparison of several mammals. *Nature* **434,** 338–345 CrossRef Medline
8. Rodriguez, A., Griffiths-Jones, S., Ashurst, J. L., and Bradley, A. (2004) Identification of mammalian microRNA host genes and transcription units. *Genome Res.* **14,** 1902–1910 CrossRef Medline
9. Berezikov, E. (2011) Evolution of microRNA diversity and regulation in animals. *Nat. Rev. Genet.* **12,** 846–860 CrossRef Medline
10. Tomari, Y., and Zamore, P. D. (2005) MicroRNA biogenesis: drosha can't cut it without a partner. *Curr. Biol.* **15,** R61–R64 CrossRef Medline
11. Yi, R., Qin, Y., Macara, I. G., and Cullen, B. R. (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev.* **17,** 3011–3016 CrossRef Medline
12. Hutvágner, G., McLachlan, J., Pasquinelli, A. E., Bálint, E., Tuschl, T., and Zamore, P. D. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293,** 834–838 CrossRef Medline
13. Gregory, R. I., Chendrimada, T. P., Cooch, N., and Shiekhattar, R. (2005) Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* **123,** 631–640 CrossRef Medline
14. Gu, S., and Chan, W. Y. (2012) Flexible and versatile as a chameleon-sophisticated functions of microRNA-199a. *Int. J. Mol. Sci.* **13,** 8449–8466 CrossRef Medline
15. Aranda, J. F., Canfrán-Duque, A., Goedeke, L., Suárez, Y., and Fernández-Hernando, C. (2015) The miR-199-dynamin regulatory axis controls receptor-mediated endocytosis. *J. Cell Sci.* **128,** 3197–3209 CrossRef Medline
16. Lee, Y. B., Bantounas, I., Lee, D. Y., Phylactou, L., Caldwell, M. A., and Uney, J. B. (2009) Twist-1 regulates the miR-199a/214 cluster during development. *Nucleic Acids Res.* **37,** 123–128 CrossRef Medline
17. Kim, S., Lee, U. J., Kim, M. N., Lee, E. J., Kim, J. Y., Lee, M. Y., Choung, S., Kim, Y. J., and Choi, Y. C. (2008) MicroRNA miR-199a* regulates the MET proto-oncogene and the downstream extracellular signal-regulated kinase 2 (ERK2). *J. Biol. Chem.* **283,** 18158–18166 CrossRef Medline
18. Fu, J., Hao, L., Tian, Y., Liu, Y., Gu, Y., and Wu, J. (2018) miR-199a-3p is involved in estrogen-mediated autophagy through the IGF-1/mTOR pathway in osteocyte-like MLO-Y4 cells. *J. Cell. Physiol.* **233,** 2292–2303 Medline
19. Wu, D., Huang, H. J., He, C. N., and Wang, K. Y. (2013) MicroRNA-199a-3p regulates endometrial cancer cell proliferation by targeting mammalian target of rapamycin (mTOR). *Int. J. Gynecol. Cancer* **23,** 1191–1197 CrossRef Medline
20. Fornari, F., Milazzo, M., Chieco, P., Negrini, M., Calin, G. A., Grazi, G. L., Pollutri, D., Croce, C. M., Bolondi, L., and Gramantieri, L. (2010) MiR-199a-3p regulates mTOR and c-Met to influence the doxorubicin sensitivity of human hepatocarcinoma cells. *Cancer Res.* **70,** 5184–5193 CrossRef Medline
21. Rane, S., He, M., Sayed, D., Vashistha, H., Malhotra, A., Sadoshima, J., Vatner, D. E., Vatner, S. F., and Abdellatif, M. (2009) Downregulation of miR-199a derepresses hypoxia-inducible factor-1α and Sirtuin 1 and recapitulates hypoxia preconditioning in cardiac myocytes. *Circ. Res.* **104,** 879–886 CrossRef Medline
22. Song, G., Zeng, H., Li, J., Xiao, L., He, Y., Tang, Y., and Li, Y. (2010) miR-199a regulates the tumor suppressor mitogen-activated protein kinase kinase kinase 11 in gastric cancer. *Biol. Pharm. Bull.* **33,** 1822–1827 CrossRef Medline
23. Gu, D. N., Jiang, M. J., Mei, Z., Dai, J. J., Dai, C. Y., Fang, C., Huang, Q., and Tian, L. (2017) MicroRNA-7 impairs autophagy-derived pools of glucose to suppress pancreatic cancer progression. *Cancer Lett.* **400,** 69–78 CrossRef Medline
24. Wang, Y., Liu, J., Liu, C., Naji, A., and Stoffers, D. A. (2013) MicroRNA-7 regulates the mTOR pathway and proliferation in adult pancreatic β-cells. *Diabetes* **62,** 887–895 CrossRef Medline
25. Sands, W. A., Page, M. M., and Selman, C. (2017) Proteostasis and ageing: insights from long-lived mutant mice. *J. Physiol.* **595,** 6383–6390 CrossRef Medline
26. Dumortier, O., Hinault, C., Gautier, N., Patouraux, S., Casamento, V., and Van Obberghen, E. (2014) Maternal protein restriction leads to pancreatic failure in offspring: role of misexpressed microRNA-375. *Diabetes* **63,** 3416–3427 CrossRef Medline
27. Eliasson, L. (2017) The small RNA miR-375: a pancreatic islet abundant miRNA with multiple roles in endocrine β cell function. *Mol. Cell. Endocrinol.* **456,** 95–101 CrossRef Medline

28. Chakraborty, C., George Priya Doss, C., and Bandyopadhyay, S. (2013) miRNAs in insulin resistance and diabetes-associated pancreatic cancer: the "minute and miracle" molecule moving as a monitor in the "genomic galaxy." *Curr. Drug Targets* **14,** 1110–1117 CrossRef Medline
29. Bolmeson, C., Esguerra, J. L., Salehi, A., Speidel, D., Eliasson, L., and Cilio, C. M. (2011) Differences in islet-enriched miRNAs in healthy and glucose-intolerant human subjects. *Biochem. Biophys. Res. Commun.* **404,** 16–22 CrossRef Medline
30. Esguerra, J. L., Bolmeson, C., Cilio, C. M., and Eliasson, L. (2011) Differential glucose-regulation of microRNAs in pancreatic islets of non-obese type 2 diabetes model Goto-Kakizaki rat. *PLoS ONE* **6,** e18613 CrossRef Medline
31. Nesca, V., Guay, C., Jacovetti, C., Menoud, V., Peyot, M. L., Laybutt, D. R., Prentki, M., and Regazzi, R. (2013) Identification of particular groups of microRNAs that positively or negatively impact on β cell function in obese models of type 2 diabetes. *Diabetologia* **56,** 2203–2212 CrossRef Medline
32. Roggli, E., Gattesco, S., Caille, D., Briet, C., Boitard, C., Meda, P., and Regazzi, R. (2012) Changes in microRNA expression contribute to pancreatic β-cell dysfunction in prediabetic NOD mice. *Diabetes* **61,** 1742–1751 CrossRef Medline
33. van de Bunt, M., Gaulton, K. J., Parts, L., Moran, I., Johnson, P. R., Lindgren, C. M., Ferrer, J., Gloyn, A. L., and McCarthy, M. I. (2013) The miRNA profile of human pancreatic islets and β-cells and relationship to type 2 diabetes pathogenesis. *PLoS ONE* **8,** e55272 CrossRef Medline
34. Alejandro, E. U., Gregg, B., Wallen, T., Kumusoglu, D., Meister, D., Chen, A., Merrins, M. J., Satin, L. S., Liu, M., Arvan, P., and Bernal-Mizrachi, E. (2014) Maternal diet-induced microRNAs and mTOR underlie β cell dysfunction in offspring. *J. Clin. Invest.* **124,** 4395–4410 CrossRef Medline
35. Roat, R., Hossain, M. M., Christopherson, J., Free, C., Jain, S., Guay, C., Regazzi, R., and Guo, Z. (2017) Identification and characterization of microRNAs associated with human β-cell loss in a mouse model. *Am. J. Transplant.* **17,** 992–1007 CrossRef Medline
36. Yan, S. T., Li, C. L., Tian, H., Li, J., Pei, Y., Liu, Y., Gong, Y. P., Fang, F. S., and Sun, B. R. (2014) miR-199a is overexpressed in plasma of type 2 diabetes patients which contributes to type 2 diabetes by targeting GLUT4. *Mol. Cell Biochem.* **397,** 45–51 CrossRef Medline
37. Bernal-Mizrachi, E., Wice, B., Inoue, H., and Permutt, M. A. (2000) Activation of serum response factor in the depolarization induction of Egr-1 transcription in pancreatic islet β-cells. *J. Biol. Chem.* **275,** 25681–25689 CrossRef Medline
38. Ishihara, H., Asano, T., Tsukuda, K., Katagiri, H., Inukai, K., Anai, M., Kikuchi, M., Yazaki, Y., Miyazaki, J. I., and Oka, Y. (1993) Pancreatic β cell line MIN6 exhibits characteristics of glucose metabolism and glucose-stimulated insulin secretion similar to those of normal islets. *Diabetologia* **36,** 1139–1145 CrossRef Medline
39. Webb, G. C., Akbar, M. S., Zhao, C., and Steiner, D. F. (2000) Expression profiling of pancreatic β cells: glucose regulation of secretory and metabolic pathway genes. *Proc. Natl. Acad. Sci. U.S.A.* **97,** 5773–5778 CrossRef Medline
40. Bijkerk, R., Esguerra, J. L. S., Ellenbroek, J. H., Au, Y. W., Hanegraaf, M. A. J., de Koning, E. J., Eliasson, L., and van Zonneveld, A. J. (2019) *In vivo* silencing of microRNA-132 reduces blood glucose and improves insulin secretion. *Nucleic Acid Ther.* **29,** 67–72 CrossRef Medline
41. Latreille, M., Hausser, J., Stützer, I., Zhang, Q., Hastoy, B., Gargani, S., Kerr-Conte, J., Pattou, F., Zavolan, M., Esguerra, J. L., Eliasson, L., Rülicke, T., Rorsman, P., and Stoffel, M. (2014) MicroRNA-7a regulates pancreatic β cell function. *J. Clin. Invest.* **124,** 2722–2735 CrossRef Medline
42. Josefsen, K., Sørensen, L. R., Buschard, K., and Birkenbach, M. (1999) Glucose induces early growth response gene (Egr-1) expression in pancreatic β cells. *Diabetologia* **42,** 195–203 CrossRef Medline
43. Eto, K., Kaur, V., and Thomas, M. K. (2007) Regulation of pancreas duodenum homeobox-1 expression by early growth response-1. *J. Biol. Chem.* **282,** 5973–5983 CrossRef Medline
44. Eto, K., Kaur, V., and Thomas, M. K. (2006) Regulation of insulin gene transcription by the immediate-early growth response gene Egr-1. *Endocrinology* **147,** 2923–2935 CrossRef Medline
45. Cheong, M. W., Kuo, L. H., Cheng, Y. N., Tsai, P. J., Ho, L. C., Tai, H. C., Chiu, W. T., Chen, S. H., Lu, P. J., Shan, Y. S., Chuang, L. M., and Tsai, Y. S. (2015) Loss of Egr-1 sensitizes pancreatic β-cells to palmitate-induced ER stress and apoptosis. *J. Mol. Med.* **93,** 807–818 CrossRef Medline
46. Miyazaki, J., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y., Oka, Y., and Yamamura, K. (1990) Establishment of a pancreatic β cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms. *Endocrinology* **127,** 126–132 CrossRef Medline
47. Blandino-Rosano, M., Barbaresso, R., Jimenez-Palomares, M., Bozadjieva, N., Werneck-de-Castro, J. P., Hatanaka, M., Mirmira, R. G., Sonenberg, N., Liu, M., Rüegg, M. A., Hall, M. N., and Bernal-Mizrachi, E. (2017) Loss of mTORC1 signalling impairs β-cell homeostasis and insulin processing. *Nat. Commun.* **8,** 16014 CrossRef Medline
48. Ardestani, A., Lupse, B., Kido, Y., Leibowitz, G., and Maedler, K. (2018) mTORC1 signaling: a double-edged sword in diabetic β cells. *Cell Metab.* **27,** 314–331 CrossRef Medline
49. Szot, G. L., Koudria, P., and Bluestone, J. A. (2007) Murine pancreatic islet isolation. *J. Vis. Exp.* **7,** e255 CrossRef Medline