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) 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 ~18–22-nucleotide-long noncoding RNAs that suppress the expression of genes by mRNA degradation, mRNA deamination, 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 (pre-miRNA) 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 3′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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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 pancreatic 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. 1A). 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. 1B). Interestingly, primiR-199a1 is the most-expressed in mouse islets, 30% higher than primiR-199a2 (Fig. 1C). In contrast, primiR-199b exhibits very low expression in islets (Fig. 1C). primiR-7a2 is 10-fold more expressed than primiR-199a1 and primiR-199a2 (Fig. 1C). The same pattern of expression was observed in MIN6 insulinoma cells: primiR-7a2 > primiR-7a1 > primiR-199a1 > primiR-199a2 > primiR-199b (Fig. 1D). 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. 1E) and male islets (Fig. 1F), expression of miR-199-3p 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. 2A). 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. 1A). 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. 2B) 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. 3A). Although a trend of increase of miR-199-5p expression was observed (1.9-fold) at 11 mm G, mouse islets in
16.6 mM G for 48 h showed a 2.7-fold increase in miR-199-5p expression (Fig. 3B).

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...
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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. S1A). 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. 5B), and prolonging KCl exposure to 6 h elevated primiR-199a2 mRNA to levels above 25 mM G (Fig. 5E). 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. 6A). 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. 6A and Fig. S3). Inhibition of glycolysis by coculturing MIN6 cells with 2-deoxy-D-glucose (2DG) and glucose showed a dose response in primiR-199a2 promoter activity to different concentrations of 2DG (Fig. 6B).

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
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 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. 7B), but transcriptional activity similar to 25 mM G was observed after 6 h (Fig. 7C). 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. 8A). A major drop in primiR-199a2 promoter activity was observed at 100 and 200 μM (Fig. 8A). 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 up-regulation of mir-199-3p by high glucose (Fig. 8B). 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
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 pri-miRs-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 KATP 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.
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
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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 insulinoma cells. Although all primiRNA levels are increased in mouse pancreatic islets and mouse mTORC1 expression, a kinase implicated in providing insights into the mechanisms responsible for glucose induction of 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 Hank’s 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 CaCl2, 1.2 mM MgCl2, 25 mM Heps, 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 miRNA 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 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 (Mm03307205_pri), primiR-7a1 (Mm03307287_pri), primiR-7a2 (Mm03307288), 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 (Mm03928990) was used as an internal control for miRNA 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 tobutamide (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 employed to assess statistically significant differences between groups, e.g. within a transfection efficiency control. All luciferase results are shown as firefly/Renilla activity and were obtained by the Dual-Luciferase® Reporter Assay System (Promega).

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