Circulating miR-375 as a Biomarker of β-Cell Death and Diabetes in Mice

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Type 1 diabetes is a progressive autoimmune disease that is largely silent in its initial stages. Yet, sensitive methods for detection of β-cell death and prediction and prevention of diabetes are lacking. Micro-RNAs (miRNAs) have been found at high concentrations in body fluids. Here in this study we sought to determine whether an islet enriched miRNA, miR-375, is a suitable blood marker to detect β-cell death and predict diabetes in mice. We measured miR-375 levels by quantitative RT-PCR in plasma samples of streptozotocin (STZ)-treated C57BL/6 mice and nonobese diabetic (NOD) mice. We also measured miR-375 levels in media samples of cytokine- or STZ-treated islets in the presence or absence of cell-death inhibitors. High-dose STZ administration dramatically increased circulating miR-375 levels, prior to the onset of hyperglycemia. Similarly, in the NOD mouse model of autoimmune diabetes, circulating miR-375 levels were significantly increased 2 weeks before diabetes onset. Moreover, cytokine- and STZ-induced cell death in isolated mouse islets produced a striking increase in extracellular miR-375 levels, which was reduced by cell death inhibitors. These data suggest that circulating miR-375 can be used as a marker of β-cell death and potential predictor of diabetes. **(*Endocrinology* 154: 603–608, 2013)**

Type 1 diabetes (T1D) is characterized by autoimmune destruction of the insulin-producing β-cells, resulting in insufficient β-cell mass and insulin secretion to control plasma glucose (1). Clinical symptoms of diabetes are usually evident once more than 70% of the β-cell population is destroyed (2). There is an unmet need for a cost-effective, reliable method to assess β-cell death in prediabetic patients, because early detection of ongoing β-cell death may offer a larger time frame for therapeutic interventions (3). Currently, the earliest sign of β-cell autoimmunity is the presence of circulating antibodies against β-cell antigens, which often can be detected months or years before the onset of clinical symptoms (4). The most common autoantibodies in prediabetic patients are directed against glutamic acid decarboxylase (GAD65), tyrosine phosphatase-like protein (IA-2), and insulin (IAA) (4). Positivity for 3 to 4 autoantibodies is associated with a risk of developing clinical T1D in the range of 60%–100% over the next 5 to 10 years. However, not everyone with autoantibodies progresses to T1D, and these measurements cannot detect ongoing β-cell death (1).

Micro-RNAs (miRNAs) are a family of endogenously produced single-stranded RNA molecules of ~22 nucleotides in length, which in the last decade have emerged as key regulators of gene expression and cell function (5). miRNA-375 (miR-375) is the first identified islet-specific miRNA (6) and the most abundant miRNA detected in the islets (7) that was shown to be important for maintaining normal α- and β-cell mass in mice (8). Recently, several miRNAs have been found in mouse and human plasma and serum at surprisingly high concentrations unlike typically labile DNA/RNA. Extracellular miRNAs appear to be by-products of dead or dying cells and remain in the extracellular space due to the high stability of the Argonaute2-miRNA complex (9) or the presence of lipoprotein complexes (10) or vesicles (11), which may confer stabil-

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Abbreviations: IFN, interferon; miRNA, micro-RNA; miR-375, miRNA-375; qRT-PCR, quantitative RT-PCR; STZ, streptozotocin; T1D, type 1 diabetes.

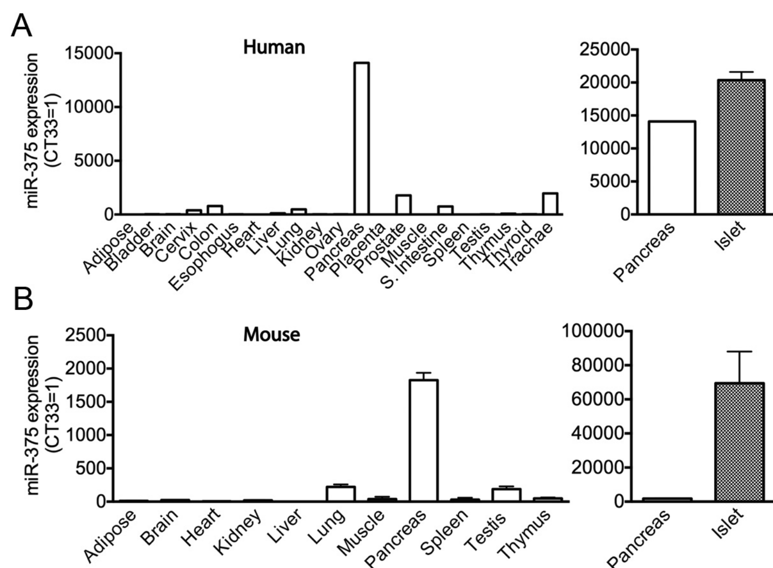

Figure 1. The abundance of miR-375 in different human and mouse tissues. A, Equal amounts of RNAs from various human tissues (each pool of 3 tissue donors, purchased from Ambion, Life Technologies) and 3 different islet preparations were reverse transcribed, and miR-375 expression was determined by qRT-PCR. miR-375 is primarily expressed in the pancreas and islets. Threshold cycle 33 (CT = 33) was arbitrarily set as 1. B, Same as in A except that the tissues were harvested from C57BL/6 mice; n = 3. n.s., not significant.

ity. Regardless of the mechanism, these miRNAs can be readily detected and quantified by PCR with sequence-specific primers, making them potentially useful as biomarkers. Given their stability and ability to be precisely quantified by PCR, we hypothesized that the most abundant, islet-enriched miRNA, miR-375, may be a sensitive biomarker of β-cell death and predictor of diabetes.

Materials and Methods

Animals

C57BL/6 male mice were obtained from the University of British Columbia Animal Care Facility and female NOD/ShiLtJ (NOD) mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Mice were fed a chow diet (2918; Research Diets, New Brunswick, New Jersey) and were housed with a 12-hour light, 12-hour dark cycle with ad libitum access to food and water. Body weight and blood glucose were measured after a 4-hour morning fast. Blood was collected from the saphenous vein with EDTA-coated capillary tubes. All procedures with animals were approved by the University of British Columbia Animal Care Committee and carried out in accordance with the Canadian Council on Animal Care guidelines. C57BL/6 mice were rendered diabetic with a single ip injection of streptozotocin (STZ) at a dose of 180 mg/kg. A subset of diabetic mice were anesthetized with inhalable isoflurane to receive an sc insulin pellet implant (Limbit; Linshin Canada, Toronto, Ontario, Canada). Diabetes was defined as 2 consecutive blood glucose measurements >10mM.

Islet isolation and culture

Human islets were obtained from the Irving K. Barber Human Islet Isolation Laboratory (Vancouver, British Columbia, Canada). Mouse islets were isolated from C57BL/6 male mice (6 weeks old) by injection of collagenase (1000 U/ml in Hanks' balanced salt solution). Pancreata were incubated at 37°C for 11 minutes, and islets were hand-picked to >95% purity. Islets were cultured in Ham's F-10 containing 0.5% BSA, 6.1mM glucose, 100 U/ml penicillin, and 100 μg/mL streptomycin. The next day, islets were plated on a 96-well plate (40 islets per well) and treated for 24 hours with a cytokine mix (IL-1β, 10 ng/mL; TNF-α, 50 ng/mL; interferon [IFN]-γ, 1000 U/mL) or 2mM STZ (in 0.1M sodium citrate, pH 4.5). When indicated, z-VAD (50μM) or PJ34 (10μM) were added 1 h before. Cell viability was assessed using the live/dead assay from Life Technologies (Burlington, Ontario, Canada).

RNA isolation and quantitative RT-PCR

The human RNA panel was purchased from Life Technologies. Mouse RNA was extracted from tissues of 8-week-old C57BL/6 mice. Tissues were homogenized with an Ultra-Turrax, and RNA was isolated using miRCURY RNA isolation kit (Exiqon, Seattle, Washington). Isolated RNA was treated with deoxyribonuclease (Life Technologies). Extracellular RNA was isolated from 25 μl plasma or 80 μl cell culture media (centrifuged for 5 minutes at 3000 × g) in the presence of an MS2 carrier RNA (Roche, Laval, Quebec) using a miRNeasy kit (QIAGEN, Mississauga, Ontario, Canada). RNA was reverse transcribed with a Universal cDNA synthesis kit (Exiqon). Quantitative RT-PCR (qRT-PCR) was performed using SYBR Green master mix (Exiqon) with LNA-based miRNA primers (Exiqon). Relative values were calculated with the ΔCT (delta threshold cycle) method.

Statistical analysis

All data are represented as mean ± SEM, and significance was set at $P \leq .05$. Mouse experiments were analyzed using a nonparametric Mann-Whitney *t* test and cell culture experiments using a parametric Student's *t* test.

Results

Circulating miR-375 is increased in STZ-treated C57BL/6 mice

We examined the tissue distribution of miR-375 by qRT-PCR and confirmed that miR-375 is primarily and abundantly expressed in the human and mouse pancreas (Figure 1, A and B). MiR-375 expression was even further enriched in islet preparations of both species compared with

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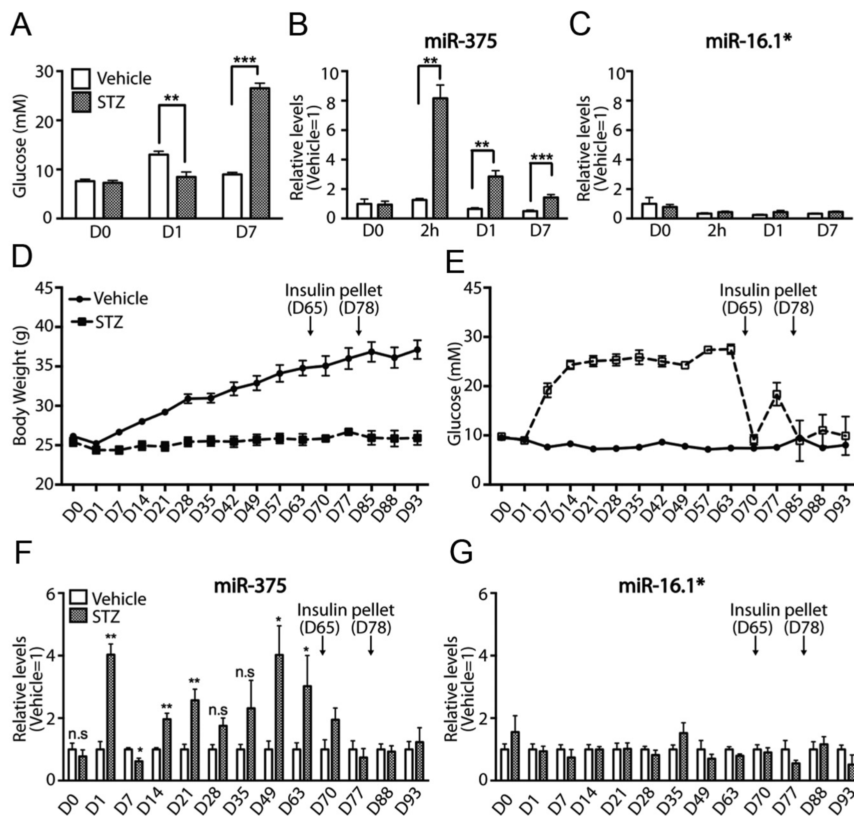

Figure 2. miR-375 levels increase in the plasma of STZ-treated mice. A–G, C57BL/6 mice were treated with a single high dose (180 mg/kg) of the β-cell toxin STZ or vehicle via ip injection. A, Blood glucose is shown. B and C, Total RNA was isolated from the plasma at each time point, and miR-375 levels (B) and miRNA-16.1* (C) were measured by qRT-PCR. D–G, Body weight and blood glucose is shown. Sixty-five days after STZ injection, mice were sc implanted with insulin pellets under isoflurane anesthesia. Plasma miR-375 levels (F) and miRNA-16.1* (G) were measured by qRT-PCR. * $P < .05$; ** $P < .01$; *** $P < .0001$; n = 4–6.

the whole pancreas, despite differences in the extent of enrichment. This difference may reflect the purity of islet fractions (hand-picked >95% pure mouse islets vs impure human islets), or that miR-375 is expressed to a greater extent in the nonislet pancreatic fraction of humans compared with mice. Regardless, these results suggested that miR-375 can indeed serve as an islet-specific marker.

We next assessed whether miR-375 could be detected in the blood and whether the levels were altered by β-cell death. We treated C57BL/6 mice with the β-cell toxin STZ and monitored blood glucose levels up to 7 days after injection (Figure 2A). STZ administration led to overt diabetes, and blood glucose levels reached 26.53 ± 1.04mM by day 7, whereas the control group was at 8.99 ± 0.39mM. Prior to diabetes, we observed the expected hypoglycemia relative to vehicle-treated controls 1 day after STZ, indicative of insulin release during β-cell death. Interestingly, plasma miR-375 levels increased by 6.8-fold compared with controls within 2 hours after STZ administration, most likely reflecting a loss of β-cell membrane integrity and cell death (Figure 2B). MiR-375 levels gradually decreased with time but were still 4.6-fold higher compared with the vehicle group on day 1 and 2.8-fold higher 7 days after STZ administration. In contrast, levels of candidate reference miRNAs, miR-16.1* and miR-16 did not change significantly with STZ treatment (Figure 2C and Supplemental Figure 1A, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). In both STZ and vehicle groups, circulating miR-16 levels were higher compared with miR-375 and miR-16.1* (Supplemental Figure 1B). Repetition of the STZ experiment in another cohort revealed a similar trend, although the effect of STZ administration on blood glucose levels was less severe. Blood glucose levels reached 19.17 ± 1.43mM by day 7 (Figure 2E), and plasma miR-375 levels increased 4.3-fold on day 1 compared with the vehicle group, returning to levels similar to that of the vehicle group by day 7 (Figure 2F). Interestingly, further monitoring of this cohort revealed a second phase increase in miR-375 levels in the diabetic group beginning on day 14 after STZ (Figure 2F). To determine whether glucose acutely regulates circulating levels of miR-375, we fasted nondiabetic C57BL/6 mice for 16 hours and assayed miR-375 in blood collected before and after refeeding the mice. Blood glucose levels increased from 4.18 ± 0.21mM to 12.61 ± 0.40mM during the meal challenge, yet there were no differences in miR-375 levels in the plasma of fasted and refed mice (n = 8, data not shown), suggesting that glucose levels do not acutely regulate miR-375 release. Next, we implanted insulin pellets into the STZ group (65 days after STZ treatment) and monitored blood glucose, miR-375, and miR16.1* levels. Insulin pellets significantly decreased fasting blood glucose levels and also miR-375 levels (Figure 2, E and F). However, miR16.1* levels were not affected by chronic hyperglycemia or insulin pellet implantation (Figure 2G). Together, these data indicate that miR-375 levels are elevated in the circulation upon acute β-cell death and chronic hyperglycemia.

Circulating miR-375 is increased in prediabetic NOD mice

We next determined whether miR-375 could be detected in the plasma of the NOD mouse model of auto-

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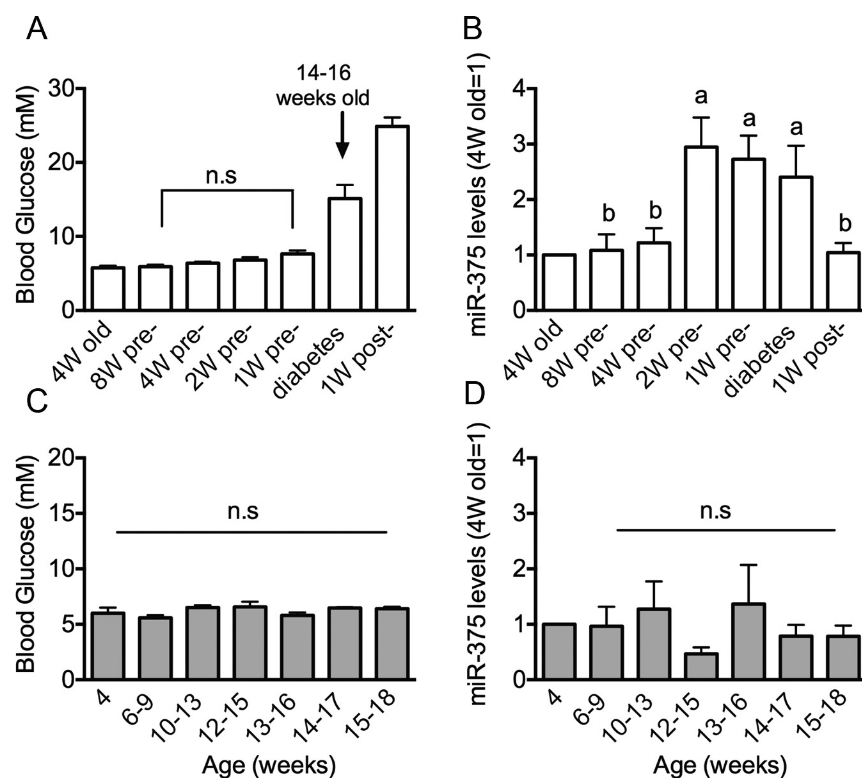

Figure 3. miR-375 levels increase in the plasma of prediabetic and diabetic NOD mice. A–D, Blood glucose (A and C) and miR-375 levels (B and D) were monitored in female diabetic (A and B) and nondiabetic (C and D) NOD mice. Total RNA was isolated from the plasma, and miR-375 levels were measured by qRT-PCR (B–D). A and B, n = 6-8; C and D, n = 6. a, Comparison with 8 weeks prediabetes; b, 1 week prediabetes ($P < .05$); n.s, not significant.

immune diabetes and used as a tool to predict diabetes. Typically, 60%–80% of female NOD mice spontaneously develop diabetes; the onset of insulitis usually occurs at 3 to 5 weeks of age, and the onset of diabetes develops from approximately 14 to 16 weeks of age (12, 13). We monitored blood glucose levels in female NOD mice until 1 week after diabetes onset (Figure 3A). Of the 34 NOD mice, 8 developed diabetes from 14-17 weeks of age (not shown). The qRT-PCR analysis of miR-375 levels in mice that became diabetic indicated that miR-375 levels significantly increased 2 weeks before the onset of diabetes (Figure 3B) prior to an increase in blood glucose levels (Figure 3A). Interestingly, miR-375 levels remained high until the diabetes onset and subsequently decreased 1 week post diabetes (Figure 3B). In age-matched nondiabetic control NOD mice (up to 19 weeks of age, blood glucose was <10mM: Figure 3C), miR-375 levels did not increase (Figure 3D). These results indicate that circulating miR-375 levels can be used as a predictive biomarker of diabetes in the NOD model of spontaneous diabetes.

MiR-375 is increased in the media of isolated islets upon cell death induction

To assess the potential mechanism of increased miR-375 levels in prediabetic samples, we isolated islets from C57BL/6 mice and incubated them with cytokines (IL-1β, TNF-α, and IFN-γ) or STZ, commonly used inducers of β-cell death, in the presence or absence of cell death inhibitors z-VAD or PJ34. Z-VAD inhibits caspase activity (14), whereas PJ34 inhibits poly (ADP-ribose) polymerase (PARP) activity (15, 16), two major mediators of cytokine and STZ-induced cell death, respectively. After 24 hours, untreated islets showed normal morphology with compact regularly shaped cells, whereas cytokine and STZ treatments led to cell disintegration with uneven morphology (Figure 4A). Z-VAD and PJ34 partially blocked these changes. Cell viability assays indicated a reduced viability of islets treated with either cytokines or STZ, which could be inhibited by the z-VAD or PJ34 cotreatments, respectively (Figure 4B). Finally, qRT-PCR analysis of media samples indicated a dramatic 24-fold increase in miR-375 levels for the islets treated with cytokines and a 20-fold increase for the STZ-treated islets, relative to controls (Figure 4C). Cytokine-induced miR-375 release could be partially inhibited with the z-VAD cotreatment yet was not significant. STZ-induced miR-375 release was significantly inhibited with the PJ34 cotreatment.

Discussion

In this report, we provide evidence that miR-375 can serve as a biomarker for detecting β-cell death and predicting diabetes in mice. We show that circulating miR-375 levels increase rapidly in vivo upon injecting mice with the β-cell toxin STZ, suggesting that there is a pool of miR-375 that is immediately released during β-cell death. This increase precedes diabetes, demonstrating the utility of our method to detect β-cell death before overt diabetes occurs. Moreover, this increase lasts about 1 week in the circulation, suggesting that miR-375 levels reflect recent β-cell loss. We also observed that chronic hyperglycemia, but not acute increases associated with meals, leads to an increase in circulating miR-375 levels, which is reversible upon insulin replacement. We speculate that the secondary increase in circulating miR-375 levels in the STZ-treated

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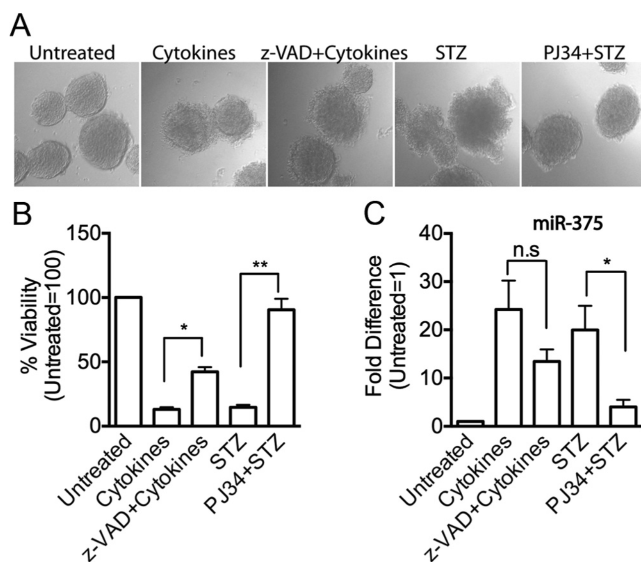

Figure 4. miR-375 levels increase in the media samples of cytokine- or STZ-treated islets. Isolated mouse islets were stimulated with cytokines (IL-1β, TNF-α, and IFN-γ) or STZ for 24 hours. One hour before, cells were pretreated with z-VAD or PJ34, as indicated. A, Phase-contrast pictures. B, Cells were stained with ethidium homodimer and calcein, and the viability was quantified by a microplate reader. C, Total RNA was isolated from the same amount of media samples, and miR-375 levels were measured by qRT-PCR. * $P < .05$; ** $P < .01$; n.s, not significant.

diabetic mice was due to glucose toxicity (17) exerted on the remaining β-cells, although we cannot exclude the possibility that other cells such as α-cells, which may expand in number after STZ treatment in mice (18), release miR-375. These observations highlight the sensitivity of our detection system and indicate that assay of circulating miR-375 by qPCR may serve as a useful tool in clinical settings to detect ongoing β-cell death.

Although high-dose STZ administration leads to acute β-cell death, autoimmune-mediated β-cell death is thought to be chronic (2). In the NOD model of autoimmune diabetes, we observed that miR-375 levels were ~3-fold increased 2 weeks before the onset of diabetes, suggesting that miR-375 can serve as a predictive biomarker of diabetes in the NOD model of autoimmune diabetes. Other earlier events such as peri-insulitis can be seen as early as 4 weeks of age in NOD mice, indicating that a rise in miR-375 levels is a relatively late marker. However, it should be noted that a major caveat of using islet inflammation as a predictive diabetes marker, aside from its invasiveness, is the high heterogeneity in disease penetrance and kinetics associated with conversion of insulitis to overt diabetes. Thus, development of other predictive, noninvasive blood markers of β-cell damage and diabetes, such as circulating miR-375 levels, is warranted.

It is not clear whether β-cells are gradually destroyed during the long preclinical phase or whether they are rapidly destroyed immediately prior to the onset of hyperglycemia in NOD mice (19, 20). Because miR-375 levels increased 2 weeks before diabetes but not earlier, our data point to a major and sudden increase in β-cell death prior to the onset of hyperglycemia rather than gradual, although a direct correlation between β-cell death and miR-375 remains to be investigated in this model. Moreover, treatment of mouse islets with cytokines and STZ clearly increased extracellular miR-375 levels, but whether different forms of β-cell death, such as apoptosis vs necrosis, affect circulating miR-375 levels also needs to be further investigated.

Prediction of diabetes in humans or mice is currently based on the presence of autoantibodies targeted to β-cell proteins (21). Future assays that can faithfully amplify low abundant signals (eg, PCR) may increase the sensitivity of disease prediction. A recent study proposed using differentially methylated circulating DNA to detect β-cell death and predict T1D (22). Although conceptually similar to our approach, quantification of miRNAs released from dying β-cells may be more sensitive (does not require nested PCR and demethylation steps) and more robust given the superior stability of miRNAs (23). However, because both methods rely on the presence of β-cells that release either methylated DNA or miR-375, these approaches should be used cautiously in the later stages of diabetes when there are few remaining β-cells. Indeed, circulating miR-375 levels may be lower in patients with T1D because most of the β-cells that contribute to the basal miR-375 levels are destroyed at the time of clinical symptoms.

It remains to be determined whether miR-375 can be used as a biomarker of β-cell death and to predict diabetes in humans similarly to what we have demonstrated in mouse models. MiR-375 is 100% conserved between mice and humans and is primarily expressed in islets in both species. MiR-375 was recently implicated in different forms of cancer and found to be elevated in the serum of patients with metastatic prostate cancer compared with patients with localized disease (24). Thus, further studies are required to determine whether this miR-375 assay with plasma samples is sensitive and specific enough for use as a single diagnostic test for detecting ongoing β-cell death in clinical settings. The combined detection of miR-375 with other islet enriched miRNAs such as miR-7 (25) may potentially improve the accuracy of detection. The quantification of miR-375 and/or other such miRNAs might have clinical utility to 1) detect ongoing β-cell death in patients with type 1 or type 2 diabetes, 2) screen diabetes-prone individuals to permit prevention or treatment, and 3) assess treatments to prevent β-cell death. Thus, our

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findings suggest that screening of miRNA profiles in prediabetic and diabetic subjects is warranted.

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S.E. wrote the first draft of the manuscript. S.E., M.M., H.D., and T.J.K. contributed to manuscript edits and revisions. S.E., M.M., H.C.D., and T.J.K. designed, directed, and interpreted experiments. S.E., M.M., H.C.D., and J.K.F. performed experiments. T.J.K. is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

Disclosure Summary: The authors have nothing to disclose.

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