Type 2 diabetes is one of the most common metabolic diseases in the world, typically characterized by absolute or relative insulin deficiency. Insulin production from pancreatic β-cells plays a vital role in maintaining glucose homeostasis in adult mammals. Factors encompassing overnutrition (glucotoxicity and lipotoxicity) and genetics (1-3) are responsible for pancreatic β-cell dysfunction, either by interfering with insulin secretion (4) or by reducing β-cell number through breaking the balance between β-cell growth and cell death (5,6). Multiple regulatory pathways connected to β-cell impairment have been widely documented, illuminating critical intracellular elements that negatively modulate β-cell mass and glucose-stimulated insulin secretion (GSIS) (7-10). A concerted action of specialized transcription factors, such as Hnf1α, Hnf1β, Pdx1, and Neurod1, is required for β-cell development, growth, and insulin gene expression. Monogenic diabetes, or maturity-onset diabetes of the young (MODY), is caused by mutations of these genes (Hnf1α-MODY, Hnf1β-MODY, Pdx1-MODY, Neurod1-MODY) (11-16). Alternatively, functional abnormalities of MODY genes contribute to nutrient overload or proinflammatory cytokine-associated β-cell failure (17-20). For example, reduced abundance and nuclear distribution of MODY transcription factors compromise insulin gene promoter activity, eventually leading to β-cell impairment (14,21-23). However, MODY gene dysregulation might involve other less well-understood molecular mechanisms.

MicroRNAs (miRNAs) are endogenous small, noncoding regulatory RNAs (~22 nt) that target the 3’ untranslated region (3’UTR) of messenger RNAs by repression of protein translation or by cleavage of miRNAs, resulting in diminished target protein production (24,25). In both plants and animals (26), miRNAs are estimated to influence ~30% of protein-coding genes (27-29). Accordingly, miRNAs have been implicated in many biological processes, including cell differentiation, cell proliferation, apoptosis, metabolism, and immune responses (30-32). However, specific functions have been identified only for a few miRNAs, and few reports have addressed these functions in β-cells (33,34).

Previously, miR-375 was found to play a significant role in endocrine pancreatic growth, development, and insulin secretion, whereby miR-375 knockout mice developed hypoglycemia with reduced β-cell mass and excess glucagon production (35). Other pancreatic β-cell regulators include miR-7, which was highly expressed in the endocrine pancreas (36), and miR34a/miR-146, which were upregulated in β-cells subject to lipotoxic stress (37). In addition, conditional deletion of Dicer1, a gene that controls miRNA processing, led to rapid hyperglycemia through the coordinated decrease in miR-24 and increase in two insulin gene transcriptional repressors, Sox6 and Bhlhe22 (38).

In this study, we identified a series of altered miRNAs in islets isolated from diabetic db/db mice compared with those of nondiabetic littermates. Among these miRNAs, miR-24 was the most enhanced. Recent publications indicated that miR-24 is consistently enriched during terminal differentiation of hematopoietic cell lines into a variety of lineages, during thymic development of naive CD8+ T cells, and during development of muscle and neuronal cells (39-41). However, the specific role of miR-24 in β-cells is unknown. Previous reports have shown that increased miR-24 inhibits cell cycle progression by directly repressing E2F2, Myc, and other cell-cycle genes in HepG2 cells, while elevated miR-24 levels induce cellular apoptosis by reducing the bcl-2 protein expression level (40,42). miR-24 is also involved in the hepatocyte nuclear factor-α-miRNA inflammatory feedback circuit to regulate hepatocellular oncogenesis (43).

Using the mouse insulin-secreting cell line (MIN6 cell) and isolated primary islets, we demonstrated that palmitate,
a free fatty acid (FFA), enhances miR-24 expression. Ec-topic expression of miR-24 resulted in failure of β-cell function. Further exploration revealed several MODY genes as direct targets of miR-24, mediating its deleterious effects. Our findings establish a new perspective on miR-24/MODY genes in regulating β-cell function, particularly as a potential mechanism for acquired obesity/FFA-induced toxicity in type 2 diabetes.

RESEARCH DESIGN AND METHODS

Cell culture and transient transfection. The mouse pancreatic β-cell line MIN6 was used between passages 16 and 32 and cultured to 70% confluence in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA) with 25 mmol/L glucose supplemented with 15% FBS (Invitrogen), 100 units/mL penicillin, 100 µg/mL streptomycin, 10 mmol/L HEPES, and 50 µmol/L β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). Cells were maintained in a Thermo tissue-culture incubator at 37°C with a 95% O2/5% CO2 atmosphere. Lipofectamine 2000 reagent (Invitrogen) was used to transfect MIN6 cells and primary islets. miRNA precursors (Ambion, Applied Biosystems, Foster City, CA) were mixed with Lipofectamine 2000 at a ratio of 10 pmol:0.5 µL miRNAs and Lipofectamine 2000. The final concentration of each miRNA in the transfection mixture was 10 pmol. MIN6 cell line manufactures’ instructions. Cotransfection experiments were performed with a ratio of 0.25 µg plasmid:10 pmol miRNAs in 48-well plates. Transfection efficiency was consistently >90% for both MIN6 cells and primary islets.

Isolation of pancreatic islets. The human pancreatic islets used in this study were from the First Affiliated Hospital of Nanjing Medical University, Nanjing, China. All animal studies were performed according to guidelines established by the Research Animal Care Committee of Nanjing Medical University. Eight- and 12-week-old C57BL/KsJ-leprdb-leprdm (db/db) mice, nonobese littermate controls, and male ICR mice (weight, 20–25 g) were purchased from the National Resource Center for Mutant Mice Model Animal Research Center of Nanjing University. Islet isolation and culturing methods have been described previously (44).

Islets isolated from db/db mice or littermate controls were collected, and an aliquot was used for miRNA extraction (400 islets/group) while the remainder was transferred to sterile 6-well plates and cultured in RPMI 1640 containing 11.1 mmol/L glucose supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin. After equilibrating for 3 h, islets were replated into 48-well plates (8 islets/well), cultured for an additional 24 h, and then used for GSIS assays. Islets isolated from ICR mice were transferred to 6-well plates and cultured overnight at 37°C. The following morning, islets were transfected with miRNA mimics and inhibitors, as described above, the islets were transfected with 50 nmol/L pre-miR-24 or pre-miR-24 for 48 h, and then replated into 48-well plates (8 islets/well) for GSIS assays. The remaining islets (~100) were used for RNA extraction.

RNAi, plasmid construction, and luciferase reporter assay. Silencing of Hnf1α and Neurod1 expression was performed using small interfering RNA (siRNA) purchased from Ribobio (Guangzhou, China) with the following sequences: Hnf1α sense, CGAAAGUUGCGAAUUGCGAATdTdT; Hnf1α antisense, UACGACUUGACCAUCUUCGdTdT; Neurod1 antisense, UACGACUUGACCAUCUUCGdTdT; Neurod1 sense, CGAAUUCGGAUGACGUAATdTdT; Neurod1 antisense, UACGACUUGACCAUCUUCGdTdT. The pGL3-basic vector (Promega, Madison, WI) was used to generate a luciferase reporter construct driven by the insulin promoter, as previously reported (40). To generate the wide-type (wt) 3’UTR-luciferase constructs of Neurod1, Kcnj8, and Kcnj11, the whole 3’UTR (1.2, 0.6, and 1.4 kb) of the mouse Neurod1 (NM_010894.2), Kcnj8 (NM_008428.4) and Kcnj11 gene (NM_01204411.1) were amplified by PCR from genomic DNA and inserted into the pMIR-REPORT Luciferase vector (Ambion) between the SacI and MluI, SpeI and HindIII, and SpeI and SacI sites, respectively. Mutant (mt) construct of Neurod1 3’UTR in the miR-24 binding site was generated using the Site-Directed Mutagenesis kit (Stratagene). All of the sequences of miR-24 MRE are from the public database (miRNA, TargetScanmouse 4.2), WT or mt sequences were obtained, synthesized, annealed, and cloned into the SpeI and HindIII sites of the pMIR-REPORT vector. Luciferase activities were measured with a dual-luciferase reporter asay system (Promega). The Firefly luciferase activity was normalized with the Renilla activity of the PRL-SV40 plasmid (Promega). The mouse Neurod1 and Hnf1α expression plasmids were constructed by inserting the full-length coding region sequences into plCMV5-myc vector or pAdTrack-CMV vector. The mouse Ccdn3 and Cdk4 expression plasmids were constructed by inserting the full-length coding region sequences into pCMV5-myc vector as well. All constructions used here were sequenced and confirmed to be correct. Sequences of primers and oligonucleotides used for cloning are provided in Supplementary Table 1.

WST-1 assay. Cell viability was determined using WST-1 assays. Briefly, the cells were seeded in 48-well plates (4 × 104 cells/well) in 200 µL culture medium and transfected with miRNAs mimics and inhibitors, as described above, for 48 h. Then, each well was supplemented with 20 µL WST-1 (Roche, São Paulo, Brazil). After 2 h, 10 µmol/L BrdU was added to each well. The cells were then processed according to the instructions provided with the labeling kit, except that in the final washing step, Hoechest 33342 (0.01 µg/mL, Sigma-Aldrich) was added to the wash reagent to stain all nuclei. Photographs of 10 random fields per coverslip were taken with the labeled cells using fluorescence microscopy. At least 500 cells were counted. The BrdU labeling index was defined as the ratio of the number of Brdu-positive nuclei to the total number of nuclei within the fields.

[H]thymidine incorporation assay. The incorporation of [3H]thymidine into islet DNA was measured as described previously (39). Briefly, after isolation and overnight culture in 24-well plates as described above, the islets were transfected with 50 nmol/L pre-miR or pre-miR-24 for 72 h. During the last 24 h, 37 kBq/mL of [methyl-3H]thymidine (37 MBq/mL, Amersham Biosciences, Little Chalfont, U.K.) was added to each well. The islets were washed twice with PBS after the culture period and sonicated in 10 mmol/L Tris-HCl 50 mL EDTA. DNA was precipitated with the addition of 10% ice-cold trichloroacetic acid and trapped by a GF/C glass-fiber disc (Whatman, Maidstone, U.K.) before being dried and radioactivity was counted after the addition of scintillation fluid.

RNA extraction, microarray, and quantitative RT-PCR analysis. MIN6 cells were cultured and treated as described above. Total RNA was extracted using Trizol reagent (Invitrogen). For miRNA microarray, total RNA samples were sent to Gene Tech (Shanghai, China) for analysis using Affymetrix GeneChip DNA microarrays. For miRNA quantification, stem-loop primers were designed with a short, single-stranded sequence complementary to the 3’-end of the miRNA, a double-stranded sequence (stem), and a loop containing the universal primer-binding sequence used for reverse primers. For mRNA determination, oligo-dT was used as reverse primers, and first-strand cDNA synthesis was performed using 1 µg total RNA (Promega). All RT-PCRs included no-template controls and RT minus controls. Quantitative (q)RT-PCR experiments were performed on an Applied Biosystems 7900HT Fast Real-Time PCR System, using a 96-well Optical 96-Channel Fast Array System Sequence Detection System (Roche, Basel, Switzerland). miRNAs were normalized with U6, and mRNA was normalized with β-actin. Sequences of the primers used are available in Supplementary Table 2. For miR-24, miR-34a, and U6, TaqMan probes purchased from Applied Biosystems (Carlsbad, CA) were used to confirm our results. Sequences of the primers used are available in Supplementary Table 2.

GSIS assay. MIN6 cells (2 × 104 cells/well) or isolated mouse islets (8 islets/well) were seeded in 48-well plates and transfected with miRNAs as above for 48 h for GSIS and potassium-stimulated insulin secretion (KSIS) assays. MIN6 cells or the islets were preincubated for 1 h in HEPES-balanced Krebs-Ringer bicarbonate buffer (KRBH) containing 2 mmol/L glucose and 1 g/L BSA. The islets or MIN6 cells were incubated for 1 h in KRBH containing basal glucose (2 mmol/L) to stimulate insulin release. After the 1 st static incubation period, supernatants were collected and frozen at −70°C for subsequent determination of insulin secretion. The insulin levels were measured using a radioimmunoassay as described previously (44).

Western immunoblotting. MIN6 cells were cultured and treated as described above and lysed with ice-cold lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 1% NP-40, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and Complete protease inhibitor (1 tablet/10 mL; Roche). After protein content determination, Western blotting was performed as described previously (44). Individual immunoblots were probed with a mouse anti-Cdk4 monoclonal antibody (mAB), mouse anti-CyclinD3 mAB, rabbit anti-CyclinD1 polyclonal antibody (pAb), rabbit anti-p16 pAb, rabbit anti-Pten pAb, and rabbit anti-parp-1 pAb (Cell Signaling, Danvers, MA) diluted 1:1000; rabbit anti-P DK1 pAb (Millipore, Billerica, MA) diluted 1:5000; rabbit anti-Hnflα pAb.
(Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:3000, rabbit anti-p27 pAb (Santa Cruz) diluted 1:1000, and goat anti-kir6.1 pAb (Santa Cruz) diluted 1:5000. Target protein levels were quantified relative to levels of control protein, mouse anti-β-tubulin mAb, and mouse anti-β-actin mAb (Sigma-Aldrich) diluted 1:500.

**Statistical analysis.** Comparisons were performed using the Student t test between pairs of groups or ANOVA for multiple group comparison. Results are presented as means ± SEM. A P value of < 0.05 was considered to be statistically significant.

**RESULTS**

**Induction of miR-24 by lipotoxicity in vivo and in vitro.** To investigate the regulation of miRNA expression in the islets of diabetic mice, we used leptin receptor–deficient db/db mice as the animal model. The 8-week-old db/db mice exhibited high body weight, hyperglycemia, loss of GSIS, and reduced insulin synthesis compared with their littermate controls (Supplementary Fig. 1A–E). qRT-PCR was performed to quantify 13 highly expressed miRNAs in isolated islets from hyperglycemic db/db mice and euglycemic littermate controls (Fig. 1A). Consistent with previous reports, miR-34a and miR-146a were approximately twofold upregulated in db/db mice, whereas miR-375 was downregulated by 70% compared with littermate controls. Among all these miRNAs, miR-24 was highly upregulated from 2.0- to 3.5-fold in 8- and 12-week-old db/db mice (Fig. 1B). In vivo, primary islets isolated from

**FIG. 1. Expression of miRNA-24 is increased in pancreatic islet cells.** A: Levels of 13 miRNAs in isolated islets from 8-week-old db/db mice (black) were analyzed relative to controls (white). Among them, miR-127, miR-21, miR-30e, miR-375, and miR-7 were statistically significantly downregulated, whereas miR-24, miR-376a, miR-146a, and miR-34a were notably upregulated (P < 0.05). No changes were observed in miR-129, miR-130a, miR-15a, and miR-181a. U6 small nuclear RNA was used as an internal control to normalize miRNA expression (*P < 0.05 or **P < 0.01 vs. control mice). B: Islets from 8- and 12-week-old db/db mice and controls were isolated, and the expression of miR-24 normalized to U6 was measured using qRT-PCR (*P < 0.05 or **P < 0.01 vs. control mice). The expression of miR-24 increased with age in the islets of db/db mice. Increasing expression levels of miR-24 and miR-34a in islets from HFD-fed mice (n = 5) compared with controls fed the standard diet (SD) (*P < 0.05 or **P < 0.01 vs. SD), palmitate-induced islets (D) (*P < 0.05 or **P < 0.01 vs. ethanol), and in palmitate-treated MIN6 cells (E) (*P < 0.05 vs. ethanol) were observed by TaqMan qRT-PCR relative to corresponding controls. U6 detected by a TaqMan probe was used as an internal control. F: Levels of miR-24 were upregulated in MIN6 cells challenged for various times (12, 24, and 48 h) with (black) or without (white) palmitate (*P < 0.05 vs. ethanol).
wild-type (WT) C57B6/J mice fed a high-fat diet (HFD) for 12 weeks became enriched with miR-24 and miR-34a compared with cohorts fed the standard diet (Fig. 1C). Isolated human pancreatic islets and MIN6 cells were treated for 48 h with or without palmitate. In vitro incubation with palmitate led to significant increases of miR-24 and miR-34a in human islets (Fig. 1D) and in MIN6 cells (Fig. 1E). Moreover, induction of miR-24 in MIN6 cells initiated at 12 h with palmitate treatment continued to rise thereafter (Fig. 1F). The elevation of miR-24 coincided with defective GSIS and KSIS (Supplementary Fig. 2A–D) and decreased cell viability (Supplementary Fig. 2E).

Enhancing miR-24 expression mediates pancreatic β-cell impairment. To identify the effects of elevated miR-24 expression, we transfected pre–miR-24 or pre-Neg miRNA precursors into MIN6 cells. The miR-24 level was significantly increased by varying pre–miR-24 concentrations from 2 to 50 nmol/L in transfections (Fig. 2A) for 48 h. The viability of MIN6 cells was significantly decreased (~72%) with transfection of miR-24 at 50 nmol/L (Fig. 2F). The cell cycle distribution analysis by flow cytometry revealed that cell proliferation was reduced starting from 10 nmol/L of transfected miR-24, mainly due to G2 phase arrest (Fig. 2C and D and Supplementary Fig. 3A). The results were confirmed by using the BrdU incorporation assay. Cells stained with BrdU and Hoechst 33342 are shown in Fig. 2E. The proportion of BrdU+ cells was diminished significantly upon overexpression of miR-24 (Fig. 2F). The ability of miR-24 to inhibit proliferation was also observed in primary mouse islets by using the [3H]thymidine incorporation assay (Fig. 2G). In all of the experiments undertaken, no changes in the level of apoptotic cells were detected (data not shown). Taken together, these observations suggest that upregulation of miR-24 expression reduces pancreatic β-cell number by inhibiting replication.

Next, overexpression of miR-24 in MIN6 cells inhibited insulin secretion induced by both glucose and potassium, whereas basal insulin secretion was not altered (Supplementary Fig. 3B and C and Fig. 2H and I). Increasing miR-24 resulted in a slight decrease in insulin promoter activity and miRNA level (Supplementary Fig. 3D and E). However, no significant change of insulin content was observed when normalized to total protein concentration (data not shown). These data demonstrated that miR-24 overexpression repressed stimulus-induced insulin secretion.

**Downregulation of target genes by miR-24 overexpression.** To understand the multiple effects caused by the elevation of miR-24, we sought to identify the regulated targets and cellular pathways by comparing mRNA microarrays transfected with pre–miR-24 or pre-Neg. In total, 351 genes were downregulated at least 1.5-fold by miR-24 overexpression (Supplementary Table 3). qRT-PCR validated 93 genes with the same results were detected with transfection of miR-24 at 50 nmol/L but not at 2 nmol/L, which was insufficient to induce cell cycle arrest. E and F: BrdU labeling was used to confirm the reduced DNA synthesis accompanying the elevation of miR-24. E: Representative images show BrdU and Hoechst stained cells, and at least 800 cells were counted. F: The BrdU labeling index is defined as the ratio of the number of BrdU+ nuclei to the total number of nuclei within the fields. G: Decreased cell proliferation was also detected in primary islets isolated from ICR mice. GSIS and KSIS assays were performed on MIN6 cells overexpressing miR-24 for 48 h, and the GSIS index (H) and KSIS index (I) were calculated. The results were similar to those in palmitate-treated cells. **P < 0.01 vs. pre-Neg.
overexpression as described above. By combining results related well with the phenotype observed after viability and decreased insulin secretion in response to (MRE) in the 3’ via directly binding to its miRNA recognition element (MRE) in the 3’ UTR of those transcripts (Supplementary Fig. 5A–E).

Hence, the reason for the downregulation of the other identified genes lacking miR-24 binding sites in their 3’UTR required further exploration. Bioinformatic analysis of their promoter sequences revealed that MODY genes, particularly Hnf1a and Neurod1, localized on promoter regions of these genes. Meanwhile, miR-24 seeds were on site of 3’UTR sequences of Hnf1a-MODY, Hnf1b-MODY, Neurod1-MODY, and Pdx1-MODY (Fig. 3A). To find out whether these four MODY genes were regulated by miR-24, WT and mutant reporter constructs of their 3’UTR, including miR-24 MREs, were cloned downstream of a luciferase reporter gene. Cotransfection with miR-24 decreased luciferase activities of the WT constructs, but no alteration of luciferase activities was observed with the mutant constructs (Fig. 3B). Only the mRNA levels of Neurod1 and Pdx1 were slightly downregulated (Fig. 3C), suggesting that miR-24 reduced their protein levels by repressing translation.

In addition to the four MODY genes, two other transcriptional factors, Cdx2 and Parp1, were also analyzed (Fig. 3A–C). To verify the luciferase reporter activity observed with the short MRE sequences, the reporter construct with the full-length 3’UTR of the Neurod1 gene was generated. As shown in Fig. 3D, a high degree of conservation is present in the aligned 3’UTR sequence of miR-24 between species (mouse and human). Overexpression of miR-24 inhibited the luciferase activity of WT Neurod1 by 33% compared with cells transfected with an empty construct, whereas no change with mutant Neurod1 was observed (Fig. 3E). The full-length 3’UTR of kcnj8 and kcnj11, each of which included a potential miR-24 MRE, was also cloned, but no significant changes in activity were detected (data not shown). These results indicated that miR-24 recognized the 3’UTR of Neurod1 and specifically repressed its expression. In agreement with results of the luciferase activity assay, Western blot analysis confirmed a reduced expression of Neurod1 in cells transfected with miR-24 for 48, 72, or 96 h (Fig. 3F). The inhibition of Neurod1 by transfected miR-24 was observed beginning at the dose of 10 nmol/L for 48 h (Fig. 3G). Together, these results suggest that miR-24 may have indirect and pleiotropic effects on many genes by modulation of MODY gene transcripts.

Analysis of functional genes in pancreatic β-cells. To further study the phenotype of MIN6 cells transfected with miR-24, protein expression levels of a series of functional genes were detected (Fig. 4A), and transcriptional factors Hnf1a, Neurod1, Pdx1, and Parp1 were significantly downregulated. Cell cycle–associated genes, such as Cdk4, Cyclind3, and p27, were repressed, whereas p15 was strikingly increased; meanwhile, no changes of Cyclind1 and Pien were observed. Kir6.1, which plays a key role in insulin secretion, was unchanged. In addition, Cyclind3, Cdk4, and p15 were altered in a time- and dose-dependent manner. mRNA levels were also changed concurrently with their protein levels (Fig. 4D). Moreover, Cyclind3 and Cdk4 were confirmed to be downregulated in primary mouse islets (Fig. 4E).

Knockdown of Hnf1a and Neurod1 mimics effects of miR-24 elevation. To assess the contribution of Hnf1a and Neurod1 to the miR-24 effects, silencing efficiencies of these molecules were examined at mRNA and protein levels (Fig. 5A and B). Combined knockdown of Hnf1a and Neurod1 contributed to the downregulation of 14 genes, including those with a 3’UTR lacking the miR-24 seed sequence, whereas the other 7 genes were not modified (Supplementary Table 4). Hnf1a and Neurod1 were both capable of decreasing BrdU incorporation. As shown by the GSIS assay, basal and stimulated levels of insulin, normalized to total protein content, were both repressed after silencing of Hnf1a and Neurod1 (Fig. 5D).

Overexpression of Hnf1a or Neurod1 rescues β-cells from effects of miR-24 elevation. To further explore the involvement of mouse Neurod1 and Hnf1a in miR-24–mediated damage, plasmids encoding these genes were cloned. Overexpression of Neurod1 in MIN6 cells was detected and found to rescue the expression of Cdk4 decreased by elevated miR-24 (Fig. 6A). Meanwhile, GSIS, KSIS, and BrdU labeling assays were done. Surprisingly, all of the impairment caused by miR-24 overexpression was rectified (Fig. 6B–D). Overexpression of Hnf1a showed the same effects as those of Neurod1 (Fig. 6E–H), whereas overexpression of both Cyclind3 and Cdk4 rescued the cell replication decreased by elevated miR-24 (Supplementary Fig. 6A–C).

Knockdown of miR-24 in islets obtained from HFD-fed mice restores normal GSIS. In vitro exposure of islets to palmitate upregulated miR-24 and impaired GSIS and KSIS. To determine whether knockdown of miR-24 could rescue islets from HFD-induced dysfunction, we isolated islets from mice fed the HFD for 10 weeks and then transfected them with an anti-miR negative control (Anti-Neg) or anti-miR-24 inhibitor (Anti–miR-24). As shown in Fig. 7A–C, HFD-fed mice gained excess weight and had elevated fasting glucose, and isolated islets demonstrated impaired GSIS compared with islets from control-fed mice. Islets obtained from HFD-fed mice were transfected with a Cy3-labeled Anti-Neg construct, and transfection efficiency was determined to be >90% when islets were counted under a fluorescent microscope (Fig. 7D). The elevated miR-24 in islets from HFD-fed mice was significantly downregulated by Anti–miR-24 transfection (Fig. 7E). Meanwhile, treatment of islets from HFD-fed mice with Anti–miR-24 restored robust GSIS compared with HFD islets treated with the Anti-Neg construct (Fig. 7F).

Oxidative stress increases miR-24 expression and inhibits miR-24 targets Neurod1 and Hnf1a. Hyperglycemia and elevated FFAs both contribute to pancreatic β-cell dysfunction through mechanisms that increase oxidative stress. Pancreatic β-cells are exquisitely sensitive to reactive oxidation because they contain low levels of the free radical scavenging enzymes catalase, glutathione peroxidase, and superoxide dismutase. We subjected MIN6 cells to two forms of oxidative stress, hyperglycemia and hydrogen peroxide (H2O2), to demonstrate the effect on...
miR-24 and downstream targets Hnf1α and Neurod1. As shown in Fig. 8A and B, hyperglycemia and H2O2 both caused an increase in miR-24 expression by ~1.6- to 1.8-fold. In Fig. 8C and D, we quantified protein levels of MODY transcription factors Hnf1α and Neurod1 using Western blot analysis. As shown, oxidative stress induced by H2O2 downregulated the Hnf1α protein level by twofold, while hyperglycemia downregulated both factors more than threefold.

**DISCUSSION**

Our findings, for the first time, demonstrate that miR-24 mediates pancreatic β-cell dysfunction, linking lipotoxicity to type 2 diabetes. This pathway seems to be conserved from mouse to human islet cells and activated in islets from diet- and obesity-induced diabetic mice. The pathogenic effects of miR-24 may occur when its level is sufficient to downregulate MODY genes, especially Neurod1.
FIG. 4. Protein and mRNA level analysis of genes are functionally associated with miR-24. A: miR-24 was transfected into MIN6 cells for 48 h, and then total protein was extracted for analysis of Hnf1a, Neurod1, Pdx1, Parp1, Cdk4, Cyclind3, p27, p15, Pten, Cycind1, kir6.1, and β-tubulin by Western blot. Protein levels remaining in cells relative to the negative control were quantitatively analyzed by Quantity One 4.2.1 (Bio-Rad).

B: A decrease of Cyclind3 was detected first at 24 h posttransfection of miR-24 (50 nmol/L) and that for Cdk4 was at 48 h. Meanwhile, the peak expression of p15 was at 24 h.

C: Transfection with miR-24 at 10 and 50 nmol/L downregulated Cyclind3 and Cdk4 protein levels and upregulated the p15 level.

D: Seven cell cycle–associated genes (CcnD1, CcnD2, CcnD3, Cdk4, p15, p21, and p27) and genes of two components of ATP-sensitive potassium (KATP) channels (Kcnj8 and Kcnj11) were determined by qRT-PCR. CcnD2, Cdk4, and p27 were decreased, whereas p15 and p21 were increased (*P < 0.05 or **P < 0.01 vs. actin).

E: mRNA levels of CcnD3 and Cdk4 were also downregulated in islets isolated from ICR mice (**P < 0.01 vs. actin).
and Hnf1a. Altering the protein level of Neurod1 or Hnf1a blocks this pathogenic effect, indicating their leading roles in the effects of miR-24.

The epidemic of type 2 diabetes is mainly ascribed to nutrient overload and genetics. Human beings, as well as rodents, are predisposed to a combination of these factors (45) that culminate in pancreatic β-cell dysfunction. A number of studies have reported that inactivation of MODY genes, including Hnf1a, Pdx1, and Neurod1, induced by diet or FFA, individually or coordinately contributes to the onset of type 2 diabetes (15,16,21). However, the modulatory mechanism of defective MODY genes was unclear. Repressing translation and increasing mRNA degradation are two recognized ways to downregulate proteins at the post-transcriptional level (41,42). Because miRNAs effectively decrease protein synthesis in both of these ways, we sought to comprehensively investigate their functions in β-cells. In the current study, besides significantly increased miR-146a and miR-34a levels, consistent with previous reports, we identified another highly enhanced miRNA, miR-24. The abundance of mature miR-24 was upregulated by palmitate in this study as well as by glucose previously (34), suggesting that miR-24 is involved in the very early onset of type 2 diabetes. In contrast, conditional inactivation of the Dicer1 protein in pancreatic β-cells, a gene that controls miRNA processing, led to rapid hyperglycemia and impaired islet insulin content that was associated with decreased miR-24 (38). Here, decreased expression of miR-24 was shown to upregulate two insulin gene transcriptional repressors, Sox6 and Bhlhe22, thereby decreasing islet insulin transcription. These contradictory findings point at a highly complex set of regulatory networks that fine-tune β-cell insulin secretion and open up the possibility that a single miRNA might provide differential input as a member of one miRNA network compared with another network. Therefore, miR-24 may play a critical role in both physiological and pathological conditions in β-cells through highly complex mechanisms that involve multiple levels of regulation.

In the current study, cDNA microarray analysis identified 351 genes downregulated by miR-24. Gene ontology enrichment analysis revealed that multiple biological processes, such as response to nutrients levels, vesicle-mediated

FIG. 5. Analysis of cellular phenotypes by silencing of Hnf1a and Neurod1. Silencing efficiencies of Hnf1a and Neurod1 were measured at mRNA (A) and protein (B) levels using qRT-PCR and Western blotting, respectively (**P < 0.01 vs. siNC). Results demonstrated that the siRNAs were effective. C: DNA synthesis was assessed by BrdU labeling. Hnf1a and Neurod1 were both able to inhibit DNA synthesis, reflecting the capacity to reduce proliferation. This inhibition was enhanced by knocking down both proteins (***P < 0.01 vs. siNC). D: GSIS assay was performed 48 h post-transfection of indicated siRNAs, and the released insulin was normalized to corresponding total insulin content. Basal and stimuli-induced insulin release were both repressed upon downregulation of Hnf1a or Neurod1 (*P < 0.05 or **P < 0.01 vs. siNC).
transport, and cell cycle control, were modulated, which agreed well with the miR-24 overexpression phenotype.

By combining computational and experimental analyses of 37 of these genes and by validating 13 direct targets of miR-24, we further found that MODY genes (Hnf1α-MODY, Hnf1β-MODY, Neurod1-MODY, and Pdx1-MODY) are directly modulated by miR-24, contributing to the decrease of the 14 miR-24 seedless genes. Thus, we have
FIG. 7. Knockdown of miR-24 expression rescued the GSIS defect in islets from HFD-fed mice. C56BL/6 mice aged 8 weeks were fed an HFD or standard diet (SD) for 10 weeks. Mice were fasted for 8 h before measuring body weight (A) and blood glucose (B). Islets from HFD-fed mice (n = 9) and SD-fed mice (n = 16) were isolated and cultured for 3 h before GSIS (C) was performed. HFD mice exhibited high body weight, hyperglycemia, and defective GSIS compared with SD mice. Secreted insulin was normalized to relative insulin content (**P < 0.01 vs. SD group). D: Islets from HFD mice were transfected with 100 nmol/L Cy3-labeled Anti-Neg. After 48 h post-transfection, photographs of Cy3-labeled islets were acquired by fluorescent microscopy and used to quantify the transfection efficiency of miRNAs in primary isolated islets. After 3-h recovery in culturing medium, islets isolated from HFD mice were transfected with 100 nmol/L Anti-Neg or Anti-miR miRNA-24 inhibitor (Anti-miR-24) for 48 h, at which time qRT-PCR (E) and GSIS (F) were carried out. U6 was used as an internal control for miRNA analysis. Insulin secretion was normalized to relative insulin content (**P < 0.01 vs. Anti-Neg + SD; #P < 0.05 or ##P < 0.01 vs. Anti-Neg + HFD).
drawn a more comprehensive profile of miR-24 downregulated genes. Existing publications generally have studied the relevance of one or two of those genes at most in the diabetic setting (14,16,21,23). Here, we demonstrated that miR-24 coordinately targets four MODY genes, representing an efficient regulatory process.

Hnf1a and Neurod1 previously were observed to be reduced in isolated primary islets under an HFD regimen (21). However, the potential mechanisms were unclear. Here, we established a connection between palmitate, miR-24 elevation, and reduction of Hnf1a and Neurod1 proteins. We described in detail the cause-and-effect relationship between the increased level of miR-24 and decreased levels of Hnf1a and Neurod1. On the basis of the existing investigations, we speculated that miR-24 was responsible for the reduction in Hnf1a and Neurod1 proteins under the HFD condition. Although β-cell dysfunction caused by upregulation of miR-24 was partially mimicked by silencing of Hnf1a and Neurod1 and recovered by ectopic expression of Neurod1 or Hnf1a, there were two phenotypic differences between these molecules. First, miR-24 elevation only caused defective insulin secretion in response to stimulus, whereas silencing Hnf1a or Neurod1 led to the deficiency in both basal and stimulated-insulin secretion. Second, although both factors decreased cell proliferation, they were associated with different cell cycle proteins; therefore, further study is needed to differentiate between the specific functions. Nevertheless, our results revealed that miR-24 affects β-cell function mainly through modulation of Hnf1a and Neurod1. Although the use of MIN6 cells might invoke a note of caution in interpreting our results, we attempted to validate our targets in both mouse and human islets whenever possible.

Treatment with protein kinase C (PKC)/mitogen-activated protein kinase activators, a reactive oxygen species (ROS) generator, or transforming growth factor-β has been shown to increase miR-24 expression (46,47). Nutrient overload and cytokines can induce ROS accumulation (20), and low levels of ROS are generated even in normal cells...
(48). Therefore, the ROS/PKC pathway is likely to be activated in diabetic and possibly prediabetic patients, thus playing a potential role in β-cell dysfunction induced by an overload of nutrients such as palmitate (23,49,50). In our studies, oxidative stress led to upregulation of miR-24 and consequent inhibition of Hnf1α and Neurod1. Moreover, we showed that knockdown of miR-24 restored GSIS in islets obtained from HFD-fed mice, suggesting that miR-24 plays a central role in translating environmental oxidative stress from FFAs to genomic regulation of β-cell insulin production through modulation of MODY transcription factors. Hence, we propose that FFA induces miR-24 expression through the ROS/PKC signaling pathway, which may occur during the very early onset of diabetes. Although we did not investigate the influence of antioxidants on miR-24 expression in this study, another report has revealed that the antioxidant drug N-acetylcysteine led to restoration of β-cell insulin production by upregulating Hnf1α protein levels (21).

In conclusion, our study reveals that miR-24 is inducible by pathogenic factors of diabetes, thus leading to β-cell failure. Importantly, our results link overnutrition and reprogramming of MODY genes to the pathological effects in type 2 diabetes. Therefore, the ROS/PKC pathway is likely to be activated in diabetic and possibly prediabetic patients, and oxidative stress from FFAs to genomic enrichment of MafA transcription factor expression. Mol Endocrinol 2011;25: 339–347.

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ACKNOWLEDGMENTS

This work was supported by grants from the National Basic Research Program of China (973 program, 2011CB504003) to X.H. and by the National Natural Science Foundation of China (81130013).

No potential conflicts of interest relevant to this article were reported.

Y.Z. researched data and participated in writing the manuscript. W.Y., H.W., Y.L., and N.Q. researched data. Y.S. and C.Z. contributed to discussion. D.B. participated in writing and editing the manuscript. X.H. provided oversight for project, participated in writing the manuscript, and edited the manuscript. X.H. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

REFERENCES

1. Wajchenberg BL. beta-Cell failure in diabetes and preservation by clinical treatment. Endocr Rev 2007;28:187–218.
2. Lindgren CM, McCarthy MI. Mechanisms of disease: genetic insights into the etiology of type 2 diabetes and obesity. Nat Clin Pract Endocrinol Metab 2008;4:156–163.
3. Leroith D, Accili D. Mechanisms of disease: using genetically altered mice to study concepts of type 2 diabetes. Nat Clin Pract Endocrinol Metab 2008;4:164–172.
4. Roccisana J, Reddy V, Vassavada RC, Gonzalez-Pertusa JA, Magnunson MA, Garcia-Ocaña A. Targeted inactivation of hepatocyte growth factor receptor c-met in beta-cells leads to defective insulin secretion and GLUT2 downregulation without alteration of beta-cell mass. Diabetes 2005;54: 2000–2012.
5. Hao E, Tyrberg B, Itkin-Ansari P, et al. Beta-cell differentiation from nonendocrine epithelial cells of the adult human pancreas. Nat Med 2006; 12:310–316.
6. Rhodes CJ. Type 2 diabetes—a matter of beta-cell life and death? Science 2005;307:389–384.
7. Cheung L, Zervou S, Mattsson G, et al. c-Myc directly induces both impaired insulin secretion and loss of β-cell mass, independently of hyperglycemia in vivo. Islets 2010;2:37–45.
8. Kim HY, Kim K. Regulation of signaling molecules associated with insulin action, insulin secretion and pancreatic β-cell mass in the hypoglycemic effects of Korean red ginseng in Goto-Kakizaki rats. J Ethnopharmacol 2012;142:53–58.
9. Yoon SY, Kim GH, Kim CH, et al. Regulation of insulin secretion and beta-cell mass by activating signal coactivator 2. Mol Cell Biol 2006;26:4553–4563.
10. Matsumura K, Chang BH, Fujiymia M, et al. Aquaporin 7 is a beta-cell protein and regulator of intralysosomal content and glycerol kinase activity, beta-cell mass, and insulin production and secretion. Mol Cell Biol 2007;27:9026–9037.
11. Fajans SS, Bell GI, Polonsky KS. Molecular mechanisms and clinical pathophysiology of maturity-onset diabetes of the young. N Engl J Med 2003;345:971–980.
12. Stride A, Hattersley AT. Different genes, different diabetes: lessons from maturity-onset diabetes of the young. Ann Med 2002;34:207–216.
13. Hunter CS, Maestro MA, Baum JC, et al. Hnf1α (MODY5) regulates β-cell-enriched Mafα transcription factor expression. Mol Endocrinol 2011;25: 339–347.
14. Wang L, Coffinier C, Thomas MK, et al. Selective deletion of the Hnflbeta (MODY5) gene in beta-cells leads to altered gene expression and defective insulin release. Endocrinology 2004;145:3941–3949.
15. Kushner JA, Ye J, Schubert M, et al. Pdx1 restores beta cell function in Irs2 knockout mice. J Clin Invest 2002;109:1193–1201.
16. Ruoho-Cabezas O, Minton JA, Kantor I, Williams D, Ellard S, Hattersley AT. Homozygous mutations in NEUROD1 are responsible for a novel syndrome of permanent neonatal diabetes and neurological abnormalities. Diabetes 2010;59:2326–2331.
17. Platt PR, Green BD. Nutrient regulation of pancreatic beta-cell function in diabetes: problems and potential solutions. Biochem Soc Trans 2006;34: 774–778.
18. Newsom P, Gaudel C, McClennagh NH. Nutrient regulation of insulin secretion and beta-cell functional integrity. Adv Exp Med Biol 2010;654:91–114.
19. Donath MY, Elshe J, Maedler K, et al. Mechanisms of beta-cell death in type 2 diabetes. Diabetes 2005;54(Suppl. 2):S108–S113.
20. Cnop M, Welsh N, Jonas JC, Jörns A, Lenzen S, Hattersley AT. Homozygous mutations in NEUROD1 are responsible for a novel syndrome of permanent neonatal diabetes and neurological abnormalities. Diabetes 2010;59:2326–2331.
21. Platt PR, Green BD. Nutrient regulation of pancreatic beta-cell function in diabetes: problems and potential solutions. Biochem Soc Trans 2006;34: 774–778.
36. Bravo-Egana V, Rosero S, Molano RD, et al. Quantitative differential expression analysis reveals miR-7 as major islet microRNA. Biochem Biophys Res Commun 2008;366:922–926
37. Lovis P, Roggli E, Laybutt DR, et al. Alterations in microRNA expression contribute to fatty acid-induced pancreatic β-cell dysfunction. Diabetes 2008;57:2728–2736
38. Melkman-Zehavi T, Oren R, Kredo-Russo S, et al. miRNAs control insulin content in pancreatic β-cells via downregulation of transcriptional repressors. EMBO J 2011;30:835–845
39. Zaidi SK, Dowdy CR, van Wijnen AJ, et al. Altered Runx1 subnuclear targeting enhances myeloid cell proliferation and blocks differentiation by activating a miR-24/MKP-7/MAPK network. Cancer Res 2009;69:8249–8255
40. Lal A, Pan Y, Navarro F, et al. miR-24-mediated downregulation of H2AX suppresses DNA repair in terminally differentiated blood cells. Nat Struct Mol Biol 2009;16:492–498
41. Neilson JR, Zheng GX, Burge CB, Sharp PA. Dynamic regulation of miRNA expression in ordered stages of cellular development. Genes Dev 2007;21:578–589
42. Lal A, Navarro F, Maher CA, et al. miR-24 Inhibits cell proliferation by targeting E2F2, MYC, and other cell-cycle genes via binding to “seedless” 3’UTR microRNA recognition elements. Mol Cell 2009;35:610–625
43. Hatziapostolou M, Polyarchou C, Aggelidou E, et al. An HNF4α-miRNA inflammatory feedback circuit regulates hepatocellular oncogenesis. Cell 2011;147:1233–1247
44. Zhu Y, Shu T, Lin Y, et al. Inhibition of the receptor for advanced glycation endproducts (RAGE) protects pancreatic β-cells. Biochem Biophys Res Commun 2011;404:159–165
45. Rossmeisl M, Rim JS, Koza RA, Kozak LP. Variation in type 2 diabetes—related traits in mouse strains susceptible to diet-induced obesity. Diabetes 2003;52:1058–1066
46. Takagi S, Nakajima M, Kida K, Yamaura Y, Fukami T, Yokoi T. MicroRNAs regulate human hepatocyte nuclear factor 4alpha, modulating the expression of metabolic enzymes and cell cycle. J Biol Chem 2010;285:4415–4422
47. Huang S, He X, Ding J, et al. Upregulation of miR-23a approximately 27a approximately 24 decreases transforming growth factor-beta-induced tumor-suppressive activities in human hepatocellular carcinoma cells. Int J Cancer 2008;123:972–978
48. Newsholme P, Rebelato E, Abdulkader F, Krause M, Carpinelli A, Curi R. Reactive oxygen and nitrogen species generation, antioxidant defenses, and β-cell function: a critical role for amino acids. J Endocrinol 2012;214:11–20
49. Ette K, Staiger H, Rieger J, et al. Protein kinase C delta activation and translocation to the nucleus are required for fatty acid-induced apoptosis of insulin-secreting cells. Diabetes 2003;52:991–997
50. Tang C, Han P, Oprescu AI, et al. Evidence for a role of superoxide generation in glucose-induced beta-cell dysfunction in vivo. Diabetes 2007;56:2722–2731