MicroRNA-30d Induces Insulin Transcription Factor MafA and Insulin Production by Targeting Mitogen-activated Protein 4 Kinase 4 (MAP4K4) in Pancreatic β-Cells*

Xiaomin Zhao§1, Ramkumar Mohan§, Sabire Özcan¹, and Xiaoping Tang§3

From the §1College of Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi 712100, China, the §2Department of Biological Sciences, Michigan Technological University, Houghton, Michigan 49931, and the §3Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, Kentucky 40536

Background: miR-30d induces insulin production, but the underlying mechanism remains unexplored.

Results: miR-30d activates MafA by targeting TNF-α-activated MAP4K4.

Conclusion: miR-30d promotes insulin production and protecting β-cell functions impaired by proinflammatory cytokines.

Significance: Overexpression of miR-30d would be beneficial in preventing the development of diabetes.

MicroRNAs (miRNAs) represent small noncoding RNAs that play a role in many diseases, including diabetes. miRNAs target genes important for pancreas development, β-cell proliferation, insulin secretion, and exocytosis. Previously, we documented that microRNA-30d (miR-30d), one of miRNAs up-regulated by glucose, induces insulin gene expression in pancreatic β-cells. Here, we found that the induction of insulin production by overexpression of miR-30d is associated with increased expression of MafA, a β-cell-specific transcription factor. Of interest, overexpression of miR-30d prevented the reduction in both MafA and insulin receptor substrate 2 (IRS2) with TNF-α exposure. Moreover, we identified that mitogen-activated protein kinase 4 (MAP4K4), a TNF-α-activated kinase, is a direct target of miR-30d. Overexpression of miR-30d protected β-cells against TNF-α suppression on both insulin transcription and insulin secretion through the down-regulation of MAP4K4 by the miR-30d. A decrease of miR-30d expression was observed in the islets of diabetic db/db mice, in which MAP4K4 expression level was elevated. Our data support the notion that miR-30d plays multiple roles in activating insulin transcription and protecting β-cell functions from impaired by proinflammatory cytokines and underscore the concept that miR-30d may represent a novel pharmacological target for diabetes intervention.

Insulin secretion and release from pancreatic β-cells represent essential processes for blood glucose homeostasis (1). Insufficient insulin production results in hyperglycemia and progression to overt diabetes (2). Insulin synthesis is primarily regulated by several key insulin transcription factors including PDX-1 (4) and MafA (3, 4). MafA is a member of the Maf family of basic-leucine zipper (bZip) transcription factors and is expressed exclusively in β-cells in the pancreas (5, 6). On the other hand, inflammatory cytokines, such as tumor necrosis factor (TNF)-α produced by pancreatic islet-infiltrating leukocytes, suppress insulin transcription factors including PDX-1 and MafA and result in a broad reduction of β-cell capacity for insulin production and release (7, 8).

MicroRNAs (miRNAs) are a new class of small, noncoding RNAs that negatively regulate gene expression by binding to the 3’-untranslated region (UTR) of target genes, leading to inhibition of protein translation or mRNA cleavage (9). Recent studies have revealed the involvement of miRNAs in specialized β-cell functions (10–13). miR-375, one of the most abundant miRNAs present in islet cells, is important for several aspects of β-cell function, such as insulin expression and secretion, as well as β-cell proliferation and adaptation to insulin resistance by intracellular homeostatic maintenance of this miRNA (14, 15). Overexpression of miR-375 suppresses glucose-induced insulin secretion and inhibition of endogenous miR-375-enhanced insulin secretion. These regulatory functions are primarily maintained through miR-375-directed targeting of Myotrophin (MTPN), a protein that plays a key role in insulin secretion and exocytosis (14, 15). In addition to targeting MTPN, miR-375 also targets 3’-phosphoinositide-dependent protein kinase 1 (PDK1) in the PI3K/protein kinase B signal cascade, the latter having a key role in insulin gene expression and β-cell growth (16). Of interest, although obese mice (ob/ob) exhibit increased expression of miR-375 (15), the diabetic Goto-Kakizaki rat appears to have a reduced level of miR-375 in the corresponding islets (16).

Besides miR-375, expression of appropriate levels of miR-124a, miR-9, and miR-29 are required for optimal insulin secre-

The abbreviations used are: PDX-1, pancreatic and duodenal homeobox1; MafA, v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A; MAP4K4, mitogen-activated protein 4 kinase 4; IRS2, insulin receptor substrate 2; miRNA, microRNA; miR-375, microRNA-375; pre-miR-30d, miR-30d RNA precursor; LNA, locked nucleic acid; Ad, adenovirus.
MicroRNA-30d Induces MafA and Insulin Production

...tion in β-cells (17–19). More recently, miR-21, miR-34a, and miR-146 were shown to function as negative regulators of insulin signaling via inhibition of insulin secretion (20). Moreover, the absolute expression of these three functionally negative miRNAs is strongly induced by inflammatory cytokines, an observation correlated with an abnormal increase of these miRNAs in the islets isolated from nonobese diabetic mice and the finding that blockade of these miRNAs prevents cytokine-induced reduction of insulin secretion (20).

An altered expression of miRNAs in other insulin-targeted organs has also been discovered in db/db or ob/ob mice. For example, the expression of miR-143 and miR-145 is markedly increased in the liver of db/db mice and diet-induced obese mice (21). miR-29 family members (miR-29a, -b, and -c) and miR-125a are up-regulated in the adipose tissue and skeletal muscles of diabetic Goto-Kakizaki rats (22, 23). The accumulating evidence suggests that loss of functionality of these miRNAs may play a role in the development of insulin resistance and type 2 diabetes, but the precise pathomechanisms remain undefined.

We have found that miR-30d, a glucose up-regulated miRNA, induces insulin gene transcription (24). In the current study, we further describe that the induction of insulin production by miR-30d is accompanied by an increased expression of MafA. Furthermore, mitogen-activated protein 4 kinase 4 (MAP4K4), a TNF-α activated kinase, was revealed as the target of miR-30d. Overexpression of miR-30d protected cells from TNF-α suppression for both insulin secretion and insulin transcription via down-regulation of MAP4K4, demonstrating a novel regulatory function of miR-30d in pancreatic β-cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and miRNA, siRNA, and Plasmid Transfection**—The insulin-secreting cell line MIN6 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen) as described (24). MIN6 cells were electroporated with 5 μg of miRNA or siRNA oligonucleotides or plasmids using the Amaxa Nucleofector system (Amaxa Inc.) according to the manufacturer’s instructions. Two days after transfection, cells were treated with low (1 mM) or high (25 mM) glucose without serum for 16 h, and then cell lysates or total RNA were prepared and subjected to analysis by Western blotting or real-time RT-PCR, respectively. For TNF-α treatment, cells were incubated with 20 ng/ml TNF-α in 25 mM glucose medium with 1% fetal bovine serum for 24 h. The following oligonucleotides have been applied in the study: miR-30d RNA precursor (pre-miR-30d), negative miRNA control precursor (pre-control), miR-30d inhibitor (anti-miR-30d), anti-miRNA negative control (anti-control), and siRNA for MAP4K4. All the oligonucleotides were purchased from Applied Biosystems.

**Mouse Islet Isolation and Culture**—Male diabetic mice (db/db, BKS.Cg-m+/+leprdb/), stock number 000642) and heterozygous control mice were 10 weeks old and obtained from The Jackson Laboratory (Bar Harbor, ME). Pancreatic islets were isolated by intraductal perfusion of collagenase V (0.5 mg/ml) (Sigma) following the protocol described (25). The purified islets were immediately processed for RNA purification and Western blot analysis. For culture, the islets were transferred into RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin. All experiments were approved by the Animal Care Committee at the Michigan Technological University.

**In Situ Hybridization**—Dissected mouse pancreas were fixed in 10% formalin for 24 h at 4 °C and then processed routinely for paraffin embedding. Tissues were cut into 5-μm sections and adhered to glass slides (Superfrost, Fisher Scientific). For in situ hybridization, sections were first deparaffinized and rehydrated with decreasing series of ethanol, washed with 0.1 M sodium phosphate buffered saline (SSC solution and incubated with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (1/1000, Roche Applied Science) overnight at 4 °C. Alkaline phosphatase reaction was carried out with 50 mg/ml nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) staining solution for 1–3 days. The LNA probes used in the study were for the detection of MAP4K4, miR-30d, let-7b, and negative control scramble. The sequence of the LNA probes is listed in Table 1.

**Adenoviral Constructs and Infection**—The recombinant adenovirus used to overexpress miR-30d (Ad-30d) was generated by subcloning miR-30d stem-loop precursor into pacAD5 miR-GFP/puro shuttle vector (Cell Biolabs). Adenoviral GFP (Ad-GFP) that does not contain a miRNA fragment was used as a control. The isolated islets were infected with adenovirus in RPMI 1640 with 2% FBS and cultured for 48 h prior to experimentation. Overexpression of miR-30d in islets was confirmed by real-time PCR analysis of miR-30d.

**Western Blots, Immunoprecipitation, and Immunofluorescence**—Western blots were performed with the following antibodies: MafA (Calbiochem), PDX-1 (Millipore), IRS2 (Cell Signaling), TBP (TATA binding protein) (Santa Cruz), and β-actin (Sigma). To detect MAP4K4 expression level, the whole-cell extracts were subjected to immunoprecipitation using anti-MAP4K4 antibody (Bethyl Laboratories) followed by immunoblot analysis with the same antibody. Anti-rabbit and anti-mouse secondary antibodies were purchased from GE Healthcare. Immunoblots were scanned on a Fuji imager, and protein levels were quantified using the ImageJ software. For immunofluorescence, MIN6 cells were fixed on 6-well chamber slides with 4% paraformaldehyde after 48 h after transfection of

| Probe          | Sequences 5’–3’          |
|----------------|--------------------------|
| mmu-miR-30d    | /5DigN/CTTCCAGTGCGGGATGTATACA |
| mmu-let-7b     | /5DigN/AACACAAACCTACTKCCCTCA  |
| Map4k4         | /5DigN/AGCATAATTATCTCCAGTTTT  |
| Scramble       | /5DigN/CTTACACCTTACAGCCCA    |
miR-30d expression plasmids. After the treatment with 0.1% Triton X-100 for the induction of cell permeability, the slides were blocked with 1% blocking goat serum and incubated with anti-MafA antibody (1/100, Calbiochem). Slides were then exposed to the Alexa Fluor 596 goat anti-rabbit IgG (1/200, Invitrogen), and images were captured using a Nikon microscope digital camera (DXM1200F).

Quantitative Real-time PCR for miRNA and mRNA—Total RNA from islets or MIN6 cells was extracted using TRIzol reagent (Invitrogen) or an RNeasy kit (Qiagen) according to the manufacturer’s instructions and treated with rDNase I (Sigma). TaqMan miRNA quantitative PCR detection system (Applied Biosystems) was used for quantification of miRNA and normalized to the relative expression of RNU19. For mRNA quantification, cDNAs were generated using a High Capacity cDNA reverse transcription kit (Applied Biosystems), and quantitative PCR was performed using TaqMan gene expression master mix or Power SYBR Green PCR master mix (Applied Biosystems). The sequences for the primers are listed in Table 2. Real-time PCR was performed on a StepOnePlus™ system (Applied Biosystems) in the following conditions: heat activation of reverse DNA polymerase at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 60 °C for 1 min. All samples were run in duplicate, and the relative amount of RNA was determined by comparison with hypoxanthine guanine phosphoribosyl transferase (HPRT) mRNA.

Luciferase Assays for miRNA Target Validation—To evaluate the predicted miR-30d complementary sites at the 3’-UTR of Map4k4, the full-length mouse Map4k4 3’-UTR was subcloned into the pRLTK vector (Promega) by PCR using mouse genomic DNA as a template. The primers were: sense, 5’-CTAGTCTAGACAGTGTGAGCTGGGATCACTGACCT-3’ and antisense, 5’-ATGACAGCAGTGTGAGCTGGGATCACTGACCT-3’. To generate Map4k4 3’-UTR mutants containing mutations in the conserved miR-30d binding site, site-directed mutagenesis was performed using the wild-type 3’-UTR as the template. For the luciferase reporter assay, pRLTK reporter constructs (2 μg) were electroporated into MIN6 cells (1 × 10⁶) with miR-30d precursor or control precursor using Amaxa (Lonza). The plasmid PGL-3 containing firefly luciferase (2 μg) was co-transfected together to normalize for transfection efficiency. Luciferase activity was measured with a Dual-Luciferase reporter assay kit (Promega) 2 days after transfection. All the related luciferase assay vectors were provided by Dr. Peter Nelson (University of Kentucky, Lexington, KY) (26).

Insulin Secretion and Content Assay—MIN6 cells were pre-incubated for 2 h in Krebs-Ringer buffer (128.8 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 2.5 mM CaCl₂, 5 mM NaHCO₃, 10 mM HEPES, 0.1% BSA) and then stimulated in the same buffer with 1 and 25 mM glucose for 1 h. The supernatant was collected and centrifuged to remove cellular debris. The total insulin content in the cells was extracted using 75% ethanol containing 1.5% (v/v) HCl. Insulin secretion and content were measured by ELISA assay using the mouse insulin immunosorbent assay kit (Mercodia). The measurement was normalized to total cellular DNA.

Statistical Analysis—Data are expressed as mean ± S.D. of three independent experiments. Statistical analyses were performed by using Student’s t test. Statistical significance was defined as p < 0.05 (marked as *) and p < 0.01 (marked as **) versus control.

RESULTS

Reduced Expression Level of miR-30d in the Islets of Diabetic Mice—We previously demonstrated that miR-30d induced glucose-regulated insulin gene transcription in the pancreatic β-cell line, Min6 (24). To investigate the change of miR-30d expression during the development of diabetes, we performed in situ hybridization in the pancreas sections of 12-week-old diabetic and heterozygous normal mice using an anti-miR-30d LNA probe (Fig. 1A). miR-30d was intensely stained in normal islets, but was weakly stained in diabetic islets. In contrast, let-7b, a widely expressed miRNA involved in apoptosis in all tissues, showed a dramatically higher expression in diabetic islets. The islets from diabetic and normal mice were isolated and further evaluated to detect miR-30d expression by real-

| TABLE 2 | Primers for real-time PCR of mRNA |
|---------|-----------------------------|
| Gene    | Forward primer                      | Reverse primer                      |
| Pre-insulin      | GGGGACGCGGCGATCCGCGAGGA  | GGGGACGCGGCGATCCGCGAAGGA  |
| Insulin 2              | CAGGAACACTCGACGACGAGAG  | TTGCAAAACGCGTCGTCGGG  |
| MafA         | AGGGCGGCGGCGATCCGCGAGGA  | CGATCCGCGGCGATCCGCGAAGGA  |
| Hprt         | TCACACACGCGGCGGATCCGCGAGGA  | GGGGACGCGGCGATCCGCGAAGGA  |
| Map4k4       | Mm00500813_m1 (Applied Biosystems) | Mm00446968_m1 (Applied Biosystems) |

FIGURE 1. Decreased expression of miR-30d in the islets of diabetic mice. A, in situ hybridization of pancreatic sections of 10-week-old heterozygous control mice (control, left panels) and diabetic mice (db/db, right panels) using digoxigenin-labeled probes for miR-30d and let-7b control. B, real-time PCR confirmed that miR-30d expression is decreased in freshly isolated islets of db/db mice at the age of 10 weeks when compared with control mice. The data are shown as mean ± S.D. of three independent experiments. *, p < 0.05, **, p < 0.01.
MicroRNA-30d Induces MafA and Insulin Production

FIGURE 2. Insulin promoter activity was activated by miR-30d. A, schematic representation of the rat insulin 1 gene promoter-luciferase construct (pRIP-Luc) in pGL3. Major important cis-regulatory elements and transcription factors that bind to these elements are indicated. B, the pRIP-Luc reporter construct was co-transfected into MIN6 cells along with pre-miR-30d, pre-control, anti-miR-30d, or anti-control oligonucleotides, respectively. The insulin promoter activities were normalized by the co-transfected pRLTK Renilla luciferase activity. Data are expressed as relative luciferase activities to the level in control. RIP, rat insulin promoter. C, the change of intracellular expression level of miR-30d was detected by real-time PCR after transfection of pre-miR-30d and anti-miR-30d. Data represent means ± S.D. of three independent experiments. **, p < 0.01.

The miR-30d precursor was transfected into the mouse pancreatic β-cell line MIN6, and the ensuing effect of miR-30d upon the expression of MafA, SREBP, and FOXO-1 was subsequently examined. We found that overexpression of miR-30d increased MafA expression 2-fold at both protein and mRNA levels (Fig. 3A), but had no effect on either FOXO-1 or SREBP protein expression. This suggested a specific role for miR-30d in the activation of MafA, a glucose-regulated and pancreatic cell-specific transcriptional activator of the insulin gene (27).

We next employed immunofluorescent microscopy to examine the effect of miR-30d on MafA expression in a single MIN6 cell. The stem-loop miR-30d precursor was introduced into an intron inside the GFP reporter gene that is under transcriptional control by the human elongation factor-1α (EF-1α) promoter (Cell Biolabs). The construct was electroporated into MIN6 cells. After 48 h, cells were stimulated with 25 mM glucose for an additional 16 h, and the expression of MafA was quantified by immunostaining. We observed significantly enhanced fluorescent immunoreactivity of MafA in cells transfected with miR30d-GFP when compared with the GFP control (Fig. 3B), confirming that miR-30d overexpression activates MafA protein expression at the single cell level.

To address the function of miR-30d in isolated primary mouse islets, we generated recombinant adenovirus overexpressing miR-30d (Ad-miR-30d) and infected it into freshly isolated primary mouse islets. Using real-time PCR analysis, we detected an increase of miR-30d in islets infected with Ad-miR-30d when compared with control islets infected with the Ad-GFP (Fig. 3C, top). Moreover, the transcription of insulin 2 gene was significantly increased in islets infected with Ad-miR-30d (Fig. 3C, bottom). The expression of MafA was consistently increased at both mRNA and protein levels (Fig. 3C, bottom). Taken together, our findings verified that miR-30d promotes insulin gene expression through activation of MafA in β-cells.

Overexpression of miR-30d Partially Restores Insulin Production and Secretion Suppressed by TNF-α—TNF-α is a pleiotropic inflammatory cytokine that decreases the capacity of β-cells to produce and release insulin. To examine the effect of TNF-α on the miR-30d-stimulated insulin and MafA expression, MIN6 cells transfected with miR-30d precursor were further treated with 20 ng/ml TNF-α for 24 h followed by quantitation of MafA expression. TNF-α treatment significantly inhibited both MafA and PDX-1 expression (Fig. 4A), whereas overexpression of miR-30d resulted in partial recovery of the expression of MafA but not PDX-1. In contrast, inhibition of miR-30d by anti-miR-30d oligonucleotides induced further suppression of MafA by TNF-α (Fig. 4A).

In β-cells, the IRS2-mediated insulin signaling pathway functions in various aspects of β-cell function. TNF-α has been shown to inhibit IRS2-mediated insulin signaling (28). To determine whether miR-30d is a general counterfactor of TNF-α action in the pathway of insulin signaling, miR-30d-overexpressed and TNF-α-treated MIN6 cells were further characterized for IRS2 expression. Overexpression of miR-30d significantly restored the level of IRS2 suppressed by TNF-α (Fig. 4B), and this restoration was likely attributable to increased IRS2 protein stability because no change in IRS2 mRNA was detected (data not shown). In contrast, inhibition of
mir-30d yielded further suppression of IRS2 by TNF-α (Fig. 4B). When compared with TNF-α exposure, IRS2 expression was not significantly altered by miR-30d under normal growth conditions. Cumulatively, our data suggest a process in which miR-30d likely targets an unidentified suppressor of IRS2 for down-regulation, and such a suppressor is activated by TNF-α because application of both TNF-α and anti-miR-30d oligonucleotides should result in a synergistic activation of this IRS2 suppressor with a concomitant down-regulation of IRS2 expression.

Because IRS2 is involved in insulin secretion (28, 29), we next examined whether miR-30d could rescue insulin secretion impaired by TNF-α via IRS2 recovery. Insulin secretion was measured in MIN6 cells transfected with miR-30d precursor after a 24-h incubation with TNF-α. We observed that insulin secretion was significantly impaired by TNF-α and that overexpression of miR-30d could only partially restore this impairment and replenish insulin secretory activity to an increased, but still overall lowered level (Fig. 4C). However, overexpression of miR-30d had little effect on insulin secretion under normal growth conditions in the absence of TNF-α (Fig. 4C) (24). Consistent with the role for miR-30d in activation of MafA and insulin gene transcription, overexpression of miR-30d increased insulin content under both normal and stressed conditions (Fig. 4D). Taken together, our findings indicate that overexpression of mir-30d can partially compensate for the defects in both insulin transcription and insulin secretion triggered by TNF-α.

miR-30d Targets MAP4K4 and Down-regulates MAP4K4 Expression in Pancreatic β-Cells—In this study, the preponderance of data indicated that miR-30d counteracts TNF-α in the protection of pancreatic β-cells from functional impairment in MafA/insulin gene expression and IRS2-mediated insulin signaling. One explanation for this counteractivity is that miR-30d targets a TNF-α-activated repressor, which suppresses MafA and/or IRS2. To search for this putative candidate repressor targeted by miR-30d, we conducted a bioinformatic analysis using miRNA online target prediction programs (TargetScan and PicTar) and screened a number of candidates that have canonical “seed” regions complementary to miR-30d at the 3’-UTR regions. These candidate miR-30d target genes included MAP4K4, Ras-related associated with diabetes (RRAD), suppressor of cytokine signaling 3 (SOCS3), and protein phosphatase 1 regulatory subunit 14c (PPP1R14C).
MAP4K4, a key downstream mediator of TNF-α action, was shown to have a very conserved seed region for the binding of miR-30d within its 3’-UTR (Fig. 5A). The minimum free energy value of this hybrid is −25.3 kcal/mol, determined by RNA hybrid analysis, which is well within the range of authentic miRNA-target pairs. This seed region can be observed in similar locations for all of the homolog genes from various species, including humans and mice, suggesting a highly conserved role in mediating the regulation of MAP4K4 by miR-30d (Fig. 5A).

To determine whether MAP4K4 is a potential target of miR-30d, pre-miR-30d transfected cells were further incubated with TNF-α for 24 h to test the effect of miR-30d on the expression of MAP4K4. Due to a low specificity of the currently available MAP4K4 antibody, the cell lysates were first immunoprecipitated with MAP4K4 antibody, and the immunoprecipitates were then employed for quantification of MAP4K4 protein by Western blotting. Introduction of the miR-30d precursor decreased the expression of MAP4K4 protein by 40% when compared with the control (Fig. 5B). Further, a moderate reduction in MAP4K4 mRNA was detected in pre-miR-30d transfected cells, indicating that the down-regulation of MAP4K4 by miR-30d was mediated via both translational repression and mRNA instability.

To further examine a potential physical interaction between miR-30d and the predicted binding site at the 3’-UTR of the Map4k4 gene, the full-length 3’-UTR of Map4k4 (Map4k4-WT) and its mutated version (Map4k4-mut), respectively, were subcloned into the 3’-UTR of a luciferase gene in a luciferase reporter vector (pRLTK, Promega). This construct was co-transfected into MIN6 cells with miR-30d precursor and a neg-

FIGURE 5. MAP4K4 is the target of miR-30d in pancreatic β-cells. A, bioinformatic prediction of the interaction between miR-30d and the 3’-UTRs of Map4k4s of various species. Mmu, mouse; Rno, rat; Has, human; Cfa, dog; and Ptr, chimpanzee. The predicted free energy of the hybrid is indicated. The mutant sequence (Map4k4-mut) is identical to the Map4k4-WT construct except for the seven point mutations (indicated in lowercase). B, miR-30d down-regulates MAP4K4 protein expression level. 48 h after transfection with pre-miR-30d or pre-control, Min6 cells were incubated with 20 ng/ml TNF-α for an additional 24 h. C, luciferase assay confirmed that miR-30d inhibited the luciferase reporter activity, in which the Map4k4-WT construct except for the seven point mutations (indicated in lowercase). **, p < 0.01.
MicroRNA-30d Induces MafA and Insulin Production

FIGURE 6. TNF-α-induced MAP4K4 inhibits insulin gene transcription and insulin signaling in pancreatic β-cells. A, MAP4K4 was activated by TNF-α (20 ng/ml) after a 24-h incubation. The expression of MAP4K4 was measured at both the protein level and mRNA level. B, silencing of MAP4K4 activated insulin gene transcription. Min6 cells were transfected with siRNAs against MAP4K4 (si-MAP4K4) or scrambled siRNA (Scr). After 48 h, total RNAs were extracted for measuring MAP4K4 and pre-insulin mRNA levels by real-time PCR. C, silencing of MAP4K4 activated the expression of MafA and IRS2, but not PDX-1. 48 h after transfection with siRNA against MAP4K4 (si-MAP4K4-1 and si-MAP4K4-2) or scrambled siRNA, cells were incubated with or without TNF-α for an additional 24 h, and cell lysates were prepared for Western blots to detect the expression of MafA, PDX-1, IRS2, and actin. Lanes #1 and #2 show two different MAP4K4 siRNAs. Data represent three independent experiments ± S.D. *, p < 0.05, **, p < 0.01.

Ative control precursor, respectively (Fig. 5C). When compared with the control, introduction of pre-miR-30d decreased the Map4k4-WT reporter activity by 60%. In contrast, point mutations in the Map4k4-miR-30d binding site (Map4k4-mut), which reduced the complementarity between miR-30d and the Map4k4 target site, abolished the repression of miR-30d on the luciferase reporter activity. However, inhibition of miR-30d did not increase the Map4k4-WT reporter activity. This is likely due to the lower level of endogenous miR-30d in Min6 cells, and anti-miR-30d only slightly reduced the miR-30d level, which had little effect on the Map4k4-WT reporter activity. Together, our data conclusively demonstrated that MAP4K4 is a direct target of miR-30d in pancreatic β-cells.

TNF-α-induced MAP4K4 Inhibits Insulin Gene Transcription and Signaling in Pancreatic β-Cells—Given that MAP4K4 was activated by TNF-α and mediated TNF-α-induced suppression of insulin secretion and signaling in rat primary β-cells (30), the identification of MAP4K4 as a target of miR-30d provides an explanation for why overexpressed miR-30d can rescue TNF-α-inhibited insulin secretion. We confirmed that both MAP4K4 protein and mRNA levels were increased in MIN6 cells treated with TNF-α for 24 h (Fig. 6A). We extended these observations by examining whether knockdown of MAP4K4 could alter insulin gene transcription using an RNA silencing approach. Silencing of MAP4K4 expression was achieved by transfecting the cells with Map4k4 siRNA (Applied Biosystems), with real-time PCR confirming a significant knockdown of MAP4K4 mRNA expression (Fig. 6B, top). As expected, incubation of MIN6 cells with TNF-α led to a significant decrease in the production of pre-insulin 2 mRNA, whereas silencing of MAP4K4 by siRNA was able to restore pre-insulin 2 mRNA expression by 60% (Fig. 6B, bottom).

To investigate how silencing of MAP4K4 could restore insulin gene transcription, we further examined the effect of silencing MAP4K4 on insulin transcription factors, including MafA and PDX-1. As shown in Fig. 6C, silencing of MAP4K4 revealed a significant increase in the expression of MafA, but not PDX-1. The data indicated that MAP4K4 triggered TNF-α suppression on insulin gene transcription via down-regulation of MafA. TNF-α treatment has been shown to induce IRS2 degradation (Fig. 4B). Of interest, knockdown of MAP4K4 by siRNA prevented the inhibitory effect of TNF-α on IRS2 expression in MIN6 cells (Fig. 6C), an observation consistent with a previous study in rat primary islets (30). Taken together, the data presented here demonstrated unequivocally that MAP4K4 negatively regulates insulin gene transcription and signaling and that silencing of MAP4K4 essentially mimicked miR-30d-targeted down-regulation of MAP4K4 in protecting β-cells from TNF-α-induced inhibition. The identification of MAP4K4 as one of miR-30d targets describes an important role for miR-30d in protecting β-cell function against the inhibitory actions of TNF-α.

Altered Expression of MAP4K4 in the Islets of Diabetic Mouse—To examine whether a change of MAP4K4 expression correlates with the change in miR-30d level in the islets of diabetic mice, a MAP4K4 antisense LNA probe was designed and used to perform an in situ hybridization analysis with pancreas sections of 10-week-old diabetic and heterozygous normal mice. In normal mice, although miR-30d was intensely stained in many cells, few islet cells could be detected with substantive MAP4K4 expression (Fig. 7A). In contrast, MAP4K4 expression was significantly increased in the diabetic islets, correlating with a significant decrease in the expression of miR-30d. The isolated islets were further analyzed for the expression of MAP4K4 by Western blot and real-time PCR, and the results demonstrated that both MAP4K4 protein and mRNA levels were increased (Fig. 7B), correlating with the decline of mir-30d in diabetic islets.

DISCUSSION

In this study, we found that miR-30d activates MafA expression and consequently promotes insulin gene transcription in pancreatic β-cells. We further discovered that miR-30d directly targets TNF-α-induced MAP4K4 and prevents the inhibitory effect of MAP4K4 on the expression of MafA and IRS2, leading to a partial recovery of TNF-α-induced suppression on insulin production and insulin secretion (Fig. 8). This discovery correlates with the fact that a decrease of miR-30d was observed in islets isolated from 10-week-old db/db mice, in which the expression of MAP4K4 was strongly increased. Our discovery is

SEPTEMBER 7, 2012 • VOLUME 287 • NUMBER 37

JOURNAL OF BIOLOGICAL CHEMISTRY 31161
supported by the recent finding that the global mature miRNA levels were significantly reduced in a H9252-cell-specific Dicer knock-out mouse, leading to the development of diabetes with a dramatic decrease in insulin production (31).

A key finding in our study is that the expression of MafA is significantly increased by overexpression of miR-30d. As a H9252-cell-specific member of the Maf family transcription factors, MafA coordinates with other insulin transcription factors and binds to the insulin promoter and promotes insulin transcription (1). Of interest, miR-30d induces insulin gene transcription through activation of MafA, but not other major insulin transcription factors, such as PDX-1 and NeuroD1. This finding indicates that the targets of miR-30d specifically repress MafA, but not other insulin transcription factors. A recent study indicates that a number of miRNAs, including miR-24/26/182/148, are also positive regulators of insulin transcription (31). Further, these miRNAs increase insulin transcription by down-regulation of several insulin transcriptional repressors, including BBHLHE22 and SOX6. Taken together, these studies demonstrate that miRNAs control the tightly regulated network of transcriptional activators and repressors that determine insulin production in β-cells.

Although MafA also activates insulin secretion (32), overexpression of miR-30d has no significant effect on insulin secretion under normal growth conditions. One potential explanation for this is that under normal growth conditions, insulin secretion is already fully activated by the glucose up-regulated endogenous miR-30d to such an extent that it cannot be further stimulated by overexpressed miR-30d. In contrast, TNF-α treatment suppresses insulin secretion to such a low level that overexpressed miR-30d can at least partially restore insulin secretion to an observable level (Fig. 4 C). These findings further imply that miR-30 may target more than one negative regulator with different affinities under different growth conditions. Identification of those potential target genes of miR-30d will lead to a more comprehensive explanation of the role(s) of miR-30d in H9252-cells.

Another pivotal observation in our work is the identification of MAP4K4 as a novel target of miR-30d under TNF-α exposure conditions. MAP4K4, a serine/threonine protein kinase, has previously been reported to induce insulin resistance in adipocytes and muscles (33–35). In pancreatic β-cells, silencing of MAP4K4 by siRNAs, mimicking the situation of miR-30d overexpression, not only partially rescues MafA and protects insulin gene transcription, but also protects insulin secretion through a sustained restoration of IRS2 and its impact upon insulin signaling (Fig. 6). The increase of IRS2 by MAP4K4

**FIGURE 7.** Altered expression levels of MAP4K4 in diabetic islets. A, in situ hybridization of pancreatic sections of 10-week-old normal control (left panels) and diabetic db/db (right panels) mice using digoxigenin-labeled probes for MAP4K4 and miR-30d. B, the expression of MAP4K4 was analyzed in freshly isolated islets of the control and db/db mice at the age of 10 weeks by using Western blots (left) and real-time PCR (right). Values shown are mean ± S.D. of three different experiments. *, p < 0.05. normal to Actin, normalized to actin.

**FIGURE 8.** A proposed mechanism for the functions of miR-30d and other interacting components in pancreatic β-cells. miR-30d plays a key role in stimulating insulin transcription (Fig. 2) and secretion (Fig. 4C) by activating MafA and IRS2 through targeting MAP4K4 and other unknown genes (red indicates a newly discovered regulatory pathway by this study). Under normal conditions, high glucose induced the production of miR-30d (based on our previous publication (24)), which further stimulates MafA (Figs. 3 and 4A), but not PDX-1 (Fig. 4A), likely by targeting unknown negative regulators of MafA. In contrast, under stress conditions, TNF-α is activated, a process that not only inhibits miR-30d, but also activates MAP4K4 (Fig. 6), the negative regulator of MafA and IRS2. At this point, miR-30d directly targets MAP4K4 and promotes the production of MafA and IRS2 (Figs. 5 and 6) and consequently insulin transcription and secretion, partially negating the reverse effects of TNF-α on β-cell functions. The function of miR-30d in activating insulin secretion is undermined by TNF-α-induced negative miRNAs miR-21, -34a, and -146 (based on previous publications) (20).
silencing has reproduced that reported earlier in rat primary β-cells (30). MafA is a highly phosphorylated protein and tightly controlled by the coordinated action of multiple kinase pathways (36–39). TNF-α-activated MAP4K4 may cause MafA phosphorylation, leading to a degradation of MafA. Consistently, in the islets of db/db mice, overproduction of proinflammatory cytokines resulted in a decrease of miR-30d expression that correlated with a sharp increase of MAP4K4, which may represent one of the primary causes for the β-cell defects in insulin transcription and secretion during the development of diabetes.

In contrast to the TNF-α-triggered reduction of miR-30d observed in the current study, a number of other miRNAs such as miR-21, miR-34a, and miR-146a are induced by proinflammatory cytokines in both MIN6 cells and human pancreatic islets (20), representing their completely different regulatory mechanisms in insulin-regulating processes. Furthermore, expressions of these miRNAs increase in islets of nonobese diabetic mice during the development of prediabetic insulitis. Blocking miR-21, miR-34a, or miR-146a function using antimiRNA oligonucleotides, respectively, in insulin-producing MIN6 cells and human pancreatic β-cells, suggest that two types of miRNAs, stimulatory and repressive, coexist in pancreatic β-cells and function interactively in insulin production and secretion. It will be of interest to dissect functional interactions of these miRNAs and their homeostatic role in diabetes.

In summary, miR-30d exerts multiple functions in activating insulin transcription and protecting β-cell functions from being impaired by proinflammatory cytokote. Given the multiple defects and complications developed in diabetic patients, the multifunctional miR-30d provides a potential novel therapeutic target in the interventional approach to the treatment of diabetes. Future studies will address the potential therapeutic role of miR-30d through a detailed dissection of miR-30d function in transgenic mice.

Acknowledgments—We thank Dr. Peter Nelson for providing luciferase reporter plasmids and Drs. K. Michael Gibson and Guiliang Tang for critical reading of this manuscript.

REFERENCES

1. Andrali, S. S., Sampley, M. L., Vanderford, N. L., and Ozcan, S. (2008) Glucose regulation of insulin gene expression in pancreatic β-cells. Biochem. J. 415, 1–10
2. Pinney, S. E., and Simmons, R. A. (2010) Epigenetic mechanisms in the development of type 2 diabetes. Trends Endocrinol. Metab. 21, 223–229
3. Ohneda, K., Ee, H., and German, M. (2000) Regulation of insulin gene transcription. Semin. Cell Biol. 11, 227–233
4. Babu, D. A., Chakrabarti, S. K., Garmey, J. C., and Miran, R. G. (2008) Pdx1 and β2/NeuroD1 participate in a transcriptional complex that mediates short-range DNA looping at the insulin gene. J. Biol. Chem. 283, 8164–8172
5. Hang, Y., and Stein, R. (2011) MaFA and MAFB activity in pancreatic β-cells. Trends Endocrinol. Metab. 22, 364–373
6. Aramata, S., Han, S. I., and Kataoka, K. (2007) Roles and regulation of transcription factor MaFA in islet β-cells. Endocr. J. 54, 659–666
7. Lawrence, M. C., Naziruddin, B., Levy, M. F., Jackson, A., and McGlynn, K. (2011) Calcineurin/nuclear factor of activated T cells and MAPK signaling induce TNF-α gene expression in pancreatic islet endocrine cells. J. Biol. Chem. 286, 1025–1036
8. Chin-Chance, C., V. Newman, M. V., Aronovitz, A., Blomieier, H., Kruger, J., Lee, E. J., and Lowe, W. L., Jr. (2006) Role of the mitogen-activated protein kinases in cytokine-mediated inhibition of insulin gene expression. J. Investig. Med. 54, 132–142
9. Bartel, D. P. (2009) MicroRNAs: target recognition and regulatory functions. Cell 136, 215–233
10. Lynn, F. C., Skews-Cox, P., Kosaka, Y., McManus, M. T., Harbe, B. D., and German, M. S. (2007) MicroRNA expression is required for pancreatic islet cell genesis in the mouse. Diabetes 56, 2938–2945
11. Gauthier, B. R., and Wollheim, C. B. (2006) MicroRNAs: “ribo-regulators” of glucose homeostasis. Nat. Med. 12, 36–38
12. Guay, C., Roggli, E., Nesca, V., Jacovetti, C., and Regazzi, R. (2011) Diabetes mellitus, a microRNA-related disease? Transl. Res. 157, 253–264
13. Tang, X., Tang, G., and Ozcan, S. (2008) Role of microRNAs in diabetes. Biochim. Biophys. Acta. 1779, 697–701
14. Poy, M. N., Eliasson, L., Krutzfeldt, J., Kwajajima, S., Ma, X., Macdonald, P. E., Pfeffer, S., Tuschil, T., Rajewsky, N., Rorsman, P., and Stoffel, M. (2004) A pancreatic islet-specific microRNA regulates insulin secretion. Nature 432, 226–230
15. Poy, M. N., Haussner, J., Trajkovski, M., Braun, M., Collins, S., Rorsman, P., Zavolon, M., and Stoffel, M. (2009) miR-375 maintains normal pancreatic α- and β-cell mass. Proc. Natl. Acad. Sci. U.S.A. 106, 5813–5818
16. El Ouaamari, A., Baroukh, M., Martens, G. A., Lebrun, P., Pipeleers, D., and van Obberghen, E. (2008) miR-375 targets 3′-phosphoinositide-dependent protein kinase-1 and regulates glucose-induced biological responses in pancreatic β-cells. Diabetes 57, 2708–2717
17. Baroukh, N., Ravier, M. A., Loder, M. K., Hill, E. V., Bounacer, A., Schirmann, R., Rutter, G. A., and Van Obberghen, E. (2007) MicroRNA-124a regulates Foxa2 expression and intracellular signaling in pancreatic β-cell lines. J. Biol. Chem. 282, 19575–19588
18. Ramachandran, D., Roy, U., Garg, S., Ghosh, S., Pathak, S., and Kolthure-Setharam, U. (2011) Sirt1 and mir-9 expression is regulated during glucose-stimulated insulin secretion in pancreatic β-islets. FEBS J 278, 1167–1174
19. Pullen, T. J., da Silva Xavier, G., Kelsey, G., and Rutter, G. A. (2011) miR-29a and miR-29b contribute to pancreatic β-cell-specific silencing of monocarboxylate transporter 1 (Mct1). Mol. Cell Biol. 31, 3182–3194
20. Roggli, E., Britan, A., Gattesco, S., Lin-Maq, N., Abderrahman, A., Meda, P., and Regazzi, R. (2010) Involvement of microRNAs in the cytotoxic effects exerted by proinflammatory cytokotyes on pancreatic β-cells. Diabetes 59, 978–986
21. Jordan, S. D., Krüger, M., Willmes, D. M., Redemann, N., Wunderlich, F. T., Bröneke, H. S., Merkwinth, C., Kashkar, H., Ollkone, V. M., Böttger, T., Braun, T., Seibler, J., and Brüning, J. C. (2011) Obesity-induced overexpression of miRNA-143 inhibits insulin-stimulated AKT activation and impairs glucose metabolism. Nat. Cell Biol. 13, 434–446
22. Andrali, S. S., Qian, Q., and Ozcan, S. (2007) Glucose mediates the translocation of NeuroD1 by O-linked glycosylation. J. Biol. Chem. 282, 15589–15596
23. Herrera, B. M., Lockstone, H. E., Taylor, J. M., Wills, Q. F., Kaisaki, P. J., Barrett, A., Camps, C., Fernandez, C., Ragoussi, J., Gauguer, D., McCarthy, M. L., and Lindgren, C. M. (2009) MicroRNA-125a is overexpressed in insulin target tissues in a spontaneous rat model of type 2 diabetes. BMC Med. Genomics 2, 54
24. Tang, X., Munipan, L., Tang, G., and Ozcan, S. (2009) Identification of glucose-regulated miRNAs from pancreatic β-cells reveals a role for miR-30d in insulin transcription. RNA 15, 287–293
25. Brissova, M., Fowler, M., Wiebe, P., Shostak, A., Shioti, M., Radhika, A., Lin, P. C., Gannon, M., and Powers, A. C. (2004) Intracellular endothelial cells contribute to revascularization of transplanted pancreatic islets. Diabetes
MicroRNA-30d Induces MafA and Insulin Production

Wang, W. X., Rajeev, B. W., Stromberg, A. I., Ren, N., Tang, G., Huang, Q., Rigoutsos, I., and Nelson, P. T. (2008) The expression of microRNA miR-107 decreases early in Alzheimer disease and may accelerate disease progression through regulation of β-site amyloid precursor protein-cleaving enzyme 1. *J Neurosci.* **28**, 1213–1223

Zhao, L., Guo, M., Matsuoka, T. A., Hagman, D. K., Parazzoli, S. D., Poitout, V., and Stein, R. (2005) The islet β-cell-enriched MafA activator is a key regulator of insulin gene transcription. *J. Biol. Chem.* **280**, 11887–11894

Gurevitch, D., Boura-Halfon, S., Isaac, R., Shahaf, G., Alberstein, M., Ronen, D., Lewis, E. C., and Zick, Y. (2010) Elimination of negative feedback control mechanisms along the insulin signaling pathway improves β-cell function under stress. *Diabetes* **59**, 2188–2197

Hennige, A. M., Burks, D. J., Ozcan, U., Kulkarni, R. N., Ye, J., Park, S., Schubert, M., Fisher, T. L., Dow, M. A., Leshan, R., Zakaria, M., Mossa-Basha, M., and White, M. F. (2003) Up-regulation of insulin receptor substrate-2 in pancreatic β-cells prevents diabetes. *J. Clin. Invest.* **112**, 1521–1532

Bouzakri, K., Ribaux, P., and Halban, P. A. (2009) Silencing mitogen-activated protein kinase 4 (MAP4K4) protects β-cells from tumor necrosis factor-α-induced decrease of IRS-2 and inhibition of glucose-stimulated insulin secretion. *J. Biol. Chem.* **284**, 27892–27898

Melkman-Zehavi, T., Oren, R., Kredo-Russo, S., Shapiro, T., Mandelbaum, A. D., Rivkin, N., Nir, T., Lennox, K. A., Behlke, M. A., Dor, Y., and Hornstein, E. (2011) miRNAs control insulin content in pancreatic β-cells via down-regulation of transcriptional repressors. *EMBO J.* **30**, 835–845

Zhang, C., Moriguchi, T., Kajihara, M., Esaki, R., Harada, A., Shimohata, H., Oishi, H., Hamada, M., Morito, N., Hasegawa, K., Kudo, T., Engel, J. D., Yamamoto, M., and Takahashi, S. (2005) MafA is a key regulator of glucose-stimulated insulin secretion. *Mol. Cell Biol.* **25**, 4969–4976

Bouzakri, K., and Zierath, J. R. (2007) MAP4K4 gene silencing in human skeletal muscle prevents tumor necrosis factor-α-induced insulin resistance. *J. Biol. Chem.* **282**, 7783–7789

Tesz, G. I., Guilherme, A., Guntur, K. V., Hubbard, A. C., Tang, X., Chawla, A., and Czech, M. P. (2007) Tumor necrosis factor α (TNF-α) stimulates Map4k4 expression through TNF-α receptor 1 signaling to c-Jun and activating transcription factor 2. *J. Biol. Chem.* **282**, 19302–19312

Guntur, K. V., Guilherme, A., Xue, L., Chawla, A., and Czech, M. P. (2010) Map4k4 negatively regulates peroxisome proliferator-activated receptor (PPAR) γ protein translation by suppressing the mammalian target of rapamycin (mTOR) signaling pathway in cultured adipocytes. *J. Biol. Chem.* **285**, 6595–6603

Guo, S., Vanderford, N. L., and Stein, R. (2010) Phosphorylation within the MafA N terminus regulates C-terminal dimerization and DNA binding. *J. Biol. Chem.* **285**, 12655–12661

Matsuoka, T. A., Kaneto, H., Miyatsuka, T., Yamamoto, T., Yamamoto, K., Kato, K., Shimomura, I., Stein, R., and Matsushima, M. (2010) Regulation of MafA expression in pancreatic β-cells in db/db mice with diabetes. *Diabetes* **59**, 1709–1720

Vanderford, N. L., Cantrell, J. E., Popa, G. J., and Ozcan, S. (2008) Multiple kinases regulate mafA expression in the pancreatic β-cell line MIN6. *Arch Biochem. Biophys.* **480**, 138–142

Eyche, A., Rocques, N., and Pouponnot, C. (2008) A new MAFia in cancer. *Nat. Rev. Cancer* **8**, 683–693