

Changes in MicroRNA Expression Contribute to Pancreatic β -Cell Dysfunction in Prediabetic NOD Mice

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During the initial phases of type 1 diabetes, pancreatic islets are invaded by immune cells, exposing β -cells to proinflammatory cytokines. This unfavorable environment results in gene expression modifications leading to loss of β -cell functions. To study the contribution of microRNAs (miRNAs) in this process, we used microarray analysis to search for changes in miRNA expression in prediabetic NOD mice islets. We found that the levels of miR-29a/b/c increased in islets of NOD mice during the phases preceding diabetes manifestation and in isolated mouse and human islets exposed to proinflammatory cytokines. Overexpression of miR-29a/b/c in MIN6 and dissociated islet cells led to impairment in glucose-induced insulin secretion. Defective insulin release was associated with diminished expression of the transcription factor Onecut2, and a consequent rise of granuphilin, an inhibitor of β -cell exocytosis. Overexpression of miR-29a/b/c also promoted apoptosis by decreasing the level of the antiapoptotic protein Mcl1. Indeed, a decoy molecule selectively masking the miR-29 binding site on Mcl1 mRNA protected insulin-secreting cells from apoptosis triggered by miR-29 or cytokines. Taken together, our findings suggest that changes in the level of miR-29 family members contribute to cytokine-mediated β -cell dysfunction occurring during the initial phases of type 1 diabetes. *Diabetes* 61:1742–1751, 2012

A fine control of the amount of insulin released by pancreatic β -cells is essential to maintain appropriate blood glucose levels. Type 1 diabetes is an autoimmune disease characterized by progressive destruction of pancreatic β -cells that, if untreated, leads to life-threatening alterations of blood glucose homeostasis and body metabolism. During the initial phases of the disease, β -cells are chronically exposed to elevated concentrations of cytokines released by the immune cells that infiltrate the islets of Langerhans and by endocrine cells themselves (1). This inflammatory environment has a deleterious impact on specialized β -cell functions, resulting in impaired insulin biosynthesis and secretion and in β -cell loss by apoptosis in the long term. A detailed understanding of the molecular events elicited by proinflammatory cytokines, and responsible for β -cell

dysfunction, is essential to pave the way to the development of new approaches for preventing and treating type 1 diabetes.

Global microarray profiling of β -cells chronically exposed to cytokines revealed that these inflammatory mediators induce major changes in gene expression, causing severe impairment in key signaling pathways (1,2). So far, most of the studies investigating the mechanisms underlying the changes in gene expression that occur in the presence of cytokines focused on transcription factors such as nuclear factor κ B (NF κ B) (3). However, there is growing evidence that another class of gene regulators, called microRNAs (miRNAs) play an important role in the control of β -cell functions, under both control and physiopathologic conditions (4–14). These short, noncoding RNA molecules bind to specific sequences of the 3'-untranslated region (3'UTR) of target mRNAs, causing message destabilization and/or translational inhibition (15,16). We recently reported that prolonged exposure of β -cells to proinflammatory cytokines leads to the induction of three miRNAs—miR-21, miR-34a, and miR-146—resulting in defective insulin secretion and apoptosis (11). We found that the level of these miRNAs is increased in the islets of NOD mice, a well characterized type 1 diabetes model (11), suggesting a potential contribution of these small RNA molecules to β -cell failure during the instauration of the disease.

In this study, we used microarray profiling to search for global changes in miRNA expression in pancreatic islets of prediabetic NOD mice. We found that, in addition to the three miRNAs mentioned above, the development of type 1 diabetes in NOD mice is associated with a strong induction in islet cells of the members of the miR-29 family. Studies performed in β -cell lines and dissociated islet cells revealed that overexpression of miR-29 to levels comparable to those observed in NOD mice leads to defective glucose-induced insulin secretion and promotes apoptosis, suggesting a contribution of this family of miRNAs to the initial phases of type 1 diabetes.

RESEARCH DESIGN AND METHODS

Chemicals. Interleukin-1 β (IL-1 β) was purchased from Sigma (Buchs, Switzerland), recombinant mouse interferon- γ (IFN- γ) from R&D Systems (Minneapolis, MN), and tumor necrosis factor- α (TNF- α) from Alexis Corporation (Lausen, Switzerland). Hoechst dye 33342 was obtained from Invitrogen (Basel, Switzerland).

Isolation and culture of pancreatic islets and MIN6 cells. The insulin-secreting cell line MIN6 clone B1 (17) was cultured at a density of 1.5×10^5 cells/cm² in DMEM-Glutamax medium (Invitrogen, Carlsbad, CA) supplemented with 15% FCS, 50 IU/mL penicillin, 50 μ g/mL streptomycin, and 70 μ mol/L β -mercaptoethanol.

Mouse pancreatic islets were isolated by collagenase digestion (18) from female NOD or C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME). After isolation, NOD mice islets were immediately processed for RNA purification. C57BL/6 mouse islet cells were dissociated by exposing the islets for 6 min to a Ca²⁺-free buffer containing 116 mmol/L NaCl, 24 mmol/L

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See accompanying commentary, p. 1661.

NaHCO₃, 5 mmol/L KCL, 1 mmol/L MgCl₂, 20 mmol/L HEPES, and 0.1% trypsin at 37°C (18). The dispersed cells were then seeded at a density of 5.5×10^4 cells/cm².

Human pancreatic islets were provided by the Cell Isolation and Transplantation Center at the University of Geneva, School of Medicine, thanks to the European Consortium for Islet Transplantation "islets for research" distribution program sponsored by the Juvenile Diabetes Research Foundation. In the current study, we used islet preparations from six donors (five women and one man) who were 56 ± 6 years old and had a BMI of 22 ± 4 kg/m². The purity of the islet preparations was $79 \pm 10\%$ and contained $51 \pm 7\%$ insulin-positive cells.

After isolation, the islets were cultured in CMRL medium (Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 100 IU/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L L-glutamine, and 250 µmol/L HEPES. Islet cell monolayers were prepared by treating the islets for 7 to 9 min with trypsin (5 mg/mL) at 37°C. Trypsinization was terminated by adding serum-containing culture medium. The cells were seeded at a density of 5.5×10^4 cells/cm².

Measurement of miRNA expression. For microarray analysis, total RNA of islets from 4- and 8-week-old NOD mice was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). Global miRNA profiling was performed by the DNA Array Facility of our institution using Agilent Technologies miRNA Gene Microarrays. The analysis permitted to determine the level of all mouse miRNAs available on the Sanger miRBase version 9.1. Quantitative assessment of the level of individual miRNAs was carried out by performing a Universal RT reaction with locked nucleic acid (LNA)-enhanced PCR primers, followed by real-time PCR (Exiqon, Vedbaek, Denmark). The results were normalized using cDNAs amplified with U6 primers in the same samples. This small nuclear RNA is not affected by cytokine treatment. Similar results were obtained when the data were normalized to miR-7.

Localization of miRNAs. To assess whether miRNAs are expressed by islet cells and/or by the intraislet immune cells, islets of 13-week-old female NOD mice were isolated by collagenase digestion and incubated for 24 h to allow spontaneous extrusion of the lymphocytes (19,20). Moreover, cryosections of pancreas of 4-, 8-, and 13-week-old NOD male mice were fixed in 4%

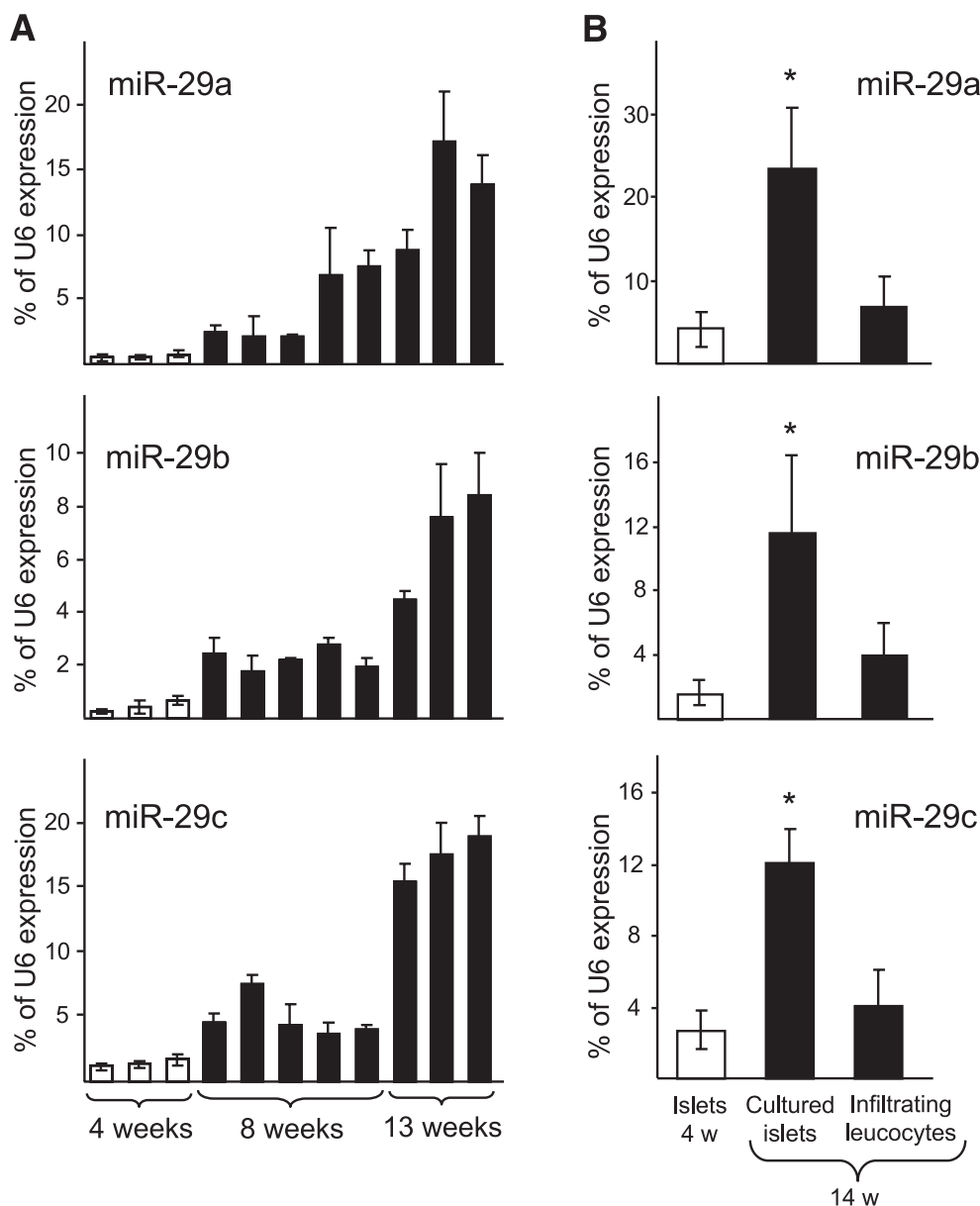


FIG. 1. The expression of the members of the miR-29 family increases with age in the pancreatic islets of NOD mice. **A:** Pancreatic islets were isolated from female NOD mice of different ages. Only animals displaying blood glucose levels within the normal range were included in the study. The levels of miR-29a/b/c were measured by qRT-PCR, expressed as percentage of U6 expression and are shown as mean \pm SEM of three to six independent experiments. **B:** Pancreatic islets were isolated from 4- and 14-week-old NOD mice. Islets of 14-week-old animals were cultured for 16 h to permit the escape of infiltrating lymphocytes. RNA was then separately extracted from the islets and the lymphocytes. The levels of miR-29a/b/c were measured by qRT-PCR, expressed as the percentage of U6 expression and are shown as mean \pm SEM of four independent experiments. * $P < 0.05$ vs. controls.

paraformaldehyde, acetylated, and processed for in situ hybridization (21) of miR-29s and miR-142-3p, using 10 nmol/L 3'-digoxigenin-labeled LNA-oligo probes (Exiqon; mmu-miR29a, MIMAT 0000535; mmu-miR29b, MIMAT 0000127; mmu-miR29c, MIMAT 0000577; mmu-miR-142-3p, MIMAT 0000155). Sections from three mice of each age were analyzed.

Adjacent sections were processed for indirect immunofluorescence staining (22) of insulin, using sheep polyclonal antibodies (Ventrex, 675), diluted 1:700; CD8a, using rat monoclonal antibodies (BD Pharmingen, 553028), diluted 1:200; granuphilin, using a rabbit polyclonal, diluted 1:250 (23); and Mcl1, using a rabbit polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:250. Incubations were performed for 2 h at room temperature (insulin, Granuphilin, Mcl1) or for 18 h at 4°C (CD8a). The sections were washed, exposed for 1 h to fluoresceinated antibodies against sheep, rat, or rabbit IgGs, counterstained with 0.03% Evans' blue, and photographed with an Axiophot fluorescence microscope (22).

Analysis of the expression of protein-coding genes. Total RNA extraction was performed with the RNeasy mini kit (Qiagen, Hilden, Germany). Conventional quantitative (q)RT-PCR was done as described (24). Real-time PCR reactions were performed on a Bio-Rad MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) using the following primers: Proinsulin, sense 5'-TGG CTT CTT CTA CAC ACC CA-3' and antisense 5'-TCT AGT TGC AGT TCT CCA-3'; 18S sense 5'-TTT CGC TCT GGT CCG TCT TG-3' and antisense 5'-TTC GGA ACT GAG GCC ATG AT-3'; and Mcl1 5'-AGA AAT GTG CTG GCT TT-3'. Samples were tested in triplicates and the results normalized using cDNA amplified with 18S primers in the same samples.

Modulation of miR-29 level and activity. To increase miRNA levels, MIN6 and primary islet cells were transiently transfected with RNA duplexes (Eurogentec, Seraing, BE) corresponding to the mature sequence of the noncoding RNA. Transient transfections of MIN6 cells and islet cells were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. For a 24-well plate, 60 pmol/well oligonucleotide duplexes were used. The transfection efficiency in MIN6 cells and dissociated islet cells evaluated with different anti-miRs and small interfering RNAs ranged between 70 and 80%. An oligonucleotide duplex corresponding to the

green fluorescent protein (GFP) sequence (5'-GAC GUA AAC GGC CAC AAG UUC-3') was used as control. Transfection of this oligonucleotide does not affect insulin content and insulin release (Supplementary Fig. 1). Transfection of the cells with small interfering GFP (or with any other control oligonucleotide duplex) increased by about 1% the fraction of cells undergoing apoptosis compared with untreated cells.

To reduce the level of miR-29, the cells were transiently transfected with an LNA miRNA inhibitor, whose sequence is 5'-CCG ATT TCA AAT GGT GCT-3' (Exiqon, Vedbaek, Denmark). To selectively prevent the binding of miR-29 to Mcl1 mRNA, without interfering with the action of the miRNA on other targets, we used a miScript Target Protector specifically designed for this purpose (Qiagen, Hilden, Germany) (25). The miScript Target Protector was transiently transfected in MIN6 cells with Lipofectamine 2000. The results were compared with those obtained with the Negative Control miScript Target Protector (Qiagen, Hilden, Germany).

Secretion assay. For the assessment of the secretory capacity, MIN6 cells (2×10^5) and dissociated mouse and human islet cells (4×10^4) plated in 24- and 96-well dishes, respectively, were transiently cotransfected with oligonucleotides corresponding to the mature miRNA sequences. After 48 h, the cells were washed and preincubated for 30 min in Krebs buffer (127 mmol/L NaCl, 4.7 mmol/L KCl, 1 mmol/L CaCl₂, 1.2 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄, 5 mmol/L NaHCO₃, 0.1% BSA, and 25 mmol/L HEPES, pH 7.4) containing 2 mmol/L glucose. The medium was discarded, and the cells were incubated 45 min in the same buffer (basal condition) or in a Krebs buffer containing 20 mmol/L glucose (stimulatory condition). After supernatants were collected, the cells were lysed in 75% ethanol and 0.55% HCl to evaluate total insulin content. The amount of insulin in the samples was assessed by ELISA (SPI-bio, Montigny-le-Bretonneux, France).

Luciferase assay. The Mcl1 3'UTR construct was generated by inserting 29 nucleotides of the 3'UTR of human Mcl1 (CAGGCTAGTCTAACC**ATGGTGC TATTATA**) corresponding to the binding sites of miR-29/a/b/c (in bold) between the SacI and MluI sites of pMir-Report (Ambion) (26). The mutant construct contained the same sequence except for a G-to-C replacement at position 21. Luciferase activities were measured with the dual-luciferase

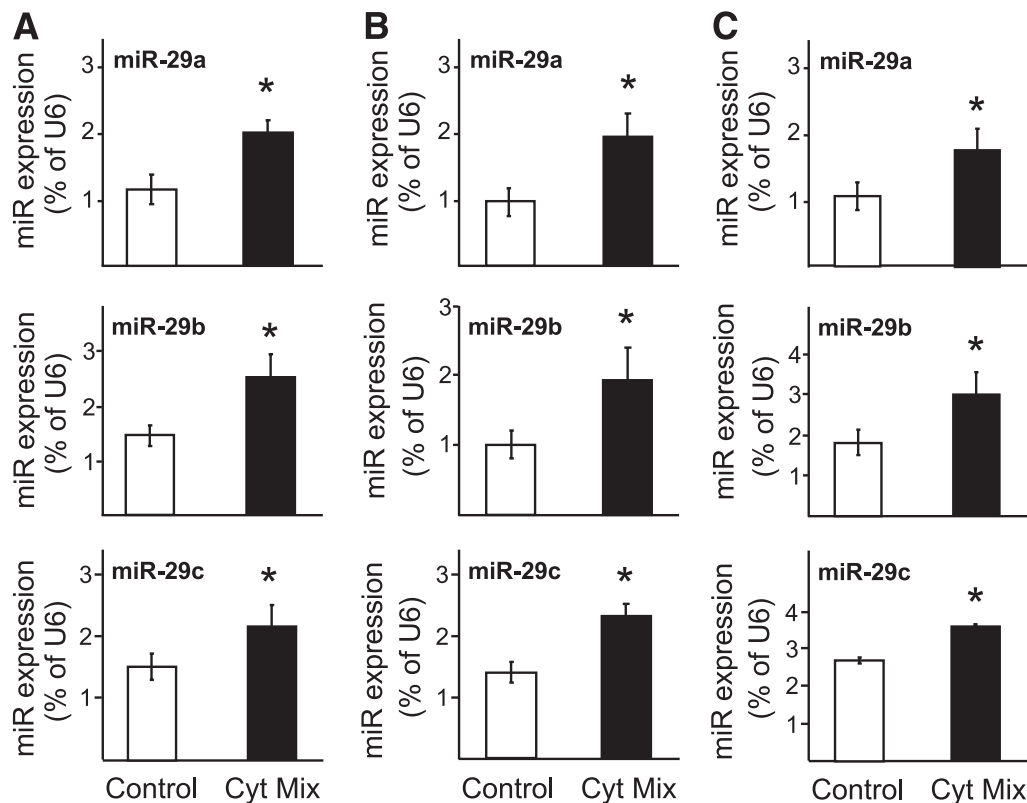


FIG. 2. Proinflammatory cytokines increase miR-29 expression. **A:** MIN6 cells were incubated for 30 h in the presence or absence of a cytokine mixture (Cyt Mix) of IL-1 β (0.1 ng/mL; 50 units/mL), plus TNF- α (10 ng/mL; 500 units/mL) and IFN- γ (30 ng/mL; 50 units/mL). miR-29a/b/c were measured by qRT-PCR and normalized to the level of U6. The results are mean \pm SD of four independent experiments. **Mouse (B) and human (C) islets** were incubated for 24 h in the presence or absence of the cytokines mixture indicated above. The levels of miR-29a/b/c were measured by qRT-PCR and normalized to the expression U6. The results are mean \pm SD of three independent experiments. * $P < 0.05$ vs. controls.

reporter assay system (Promega, Madison, WI). The *Firefly* luciferase activity was normalized for transfection efficiency with the SV40-driven *Renilla* activity generated by the psiCHECK-1 vector (Promega).

Protein extraction and Western blotting. MIN6 cells or dissociated mouse islet cells were incubated for 15 min on ice in lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 0.5% Triton X-100, 137.5 mmol/L NaCl, 10% glycerol, 1 mmol/L sodium vanadate, 50 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 5 mmol/L EDTA) and Protease Inhibitors Cocktail (Sigma, St. Louis, MO) and then sonicated. The homogenate was centrifuged at 12,000 rpm for 1 min and the supernatant collected for further analysis. Fifty micrograms of the protein extracts were separated on acrylamide gels and transferred on polyvinylidene fluoride membranes. The membranes were incubated overnight at 4°C with primary antibodies. Immunoreactive bands were visualized by chemiluminescence (Amersham Biosciences) after incubation with horseradish peroxidase-coupled secondary antibodies for 1 h at room temperature. The antibodies against Onecut2 (amino acid 36-311) and granuphilin have been previously described (23,27). The antibody directed against Syntaxin 1a (S0664) was purchased from Sigma (Buchs, Switzerland), and that against Mcl-1 (sc-819) was from Santa Cruz Biotechnology. The antibody against actin was from Chemicon International (Temecula, CA).

Evaluation of the fraction of cells undergoing apoptosis. For the assessment of apoptosis, MIN6 cells (1×10^5) or dissociated islet cells (8×10^4) were plated in 24-well dishes and transiently transfected with oligonucleotides leading to miRNA overexpression. Apoptosis was assessed 2 days later by scoring the cells displaying pyknotic nuclei upon Hoechst dye 33342 staining. The experiments were carried out blindly, and at least 800 cells per condition were analyzed. To distinguish between apoptosis and necrosis, the cells were seeded on glass coverslips coated with 2 mg/mL poly-L-lysine (Sigma, Buchs, Switzerland) and 33.2 μ g/mL laminin (Invitrogen, Basel, Switzerland). Cells undergoing apoptosis or necrosis were identified using the Annexin V-EGFP Apoptosis Kit (Biovision, Mountain View, CA) 72 h after the transfection.

Statistical analysis. Statistical differences were tested by ANOVA. The experiments including more than two groups were first analyzed by ANOVA, and multiple comparisons of the means were then carried out using the post hoc Dunnett test, with a discriminating *P* value of 0.05.

RESULTS

We compared the global miRNA expression profile of pancreatic islets of 4- and 8-week-old NOD mice. In agreement with our previous findings (11), the islets of 8-week-old mice displayed an increase in the expression of miR-21, miR-34a, and miR-146a/b (Supplementary Table 1). In addition, we detected significant alterations in the level of several other miRNAs, including miR-142-3p, miR-142-5p, miR-150, miR-155, and miR-216a, which are almost undetectable in the islets of 4-week-old NOD mice (Supplementary Fig. 2 and 3). Because these miRNAs are expected to be particularly abundant in immune cells (28), their expression was evaluated in islets of 14-week-old, prediabetic NOD mice, which were cultured to allow for the escaping of the infiltrated lymphocytes (29). These miRNAs were 100- to 1,000-fold more abundant in the immune cells that had escaped the islets than in islet cells (Supplementary Fig. 3). In situ hybridization combined with immunofluorescence staining for insulin and CD8a (the hybridization and the immunostaining were performed on sequential sections of the very same pancreases), confirmed that most of the miR-142-3p is expressed in immune cells and not in insulin-producing cells (Supplementary Fig. 4, *top panels*). We concluded that the increase in the latter miRNA in the islet preparations of aging NOD animals likely reflected mainly the presence of immune cells; for this reason, this group of miRNAs was not investigated any further in this study.

Among the several other miRNAs that were differentially expressed between 4- and 8-week-old animals, we noticed the members of the miR-29 family (miR-29a/b/c) that are among the most abundant miRNAs present in normal β -cells (30) (Supplementary Table 1). These

miRNAs have been shown to downregulate the expression of the β -cell disallowed gene, monocarboxylase transporter 1 (31,32) and to contribute to insulin resistance in insulin target tissues in GK rats (33). Analysis by qPCR confirmed upregulation of miR-29a/b/c in the islets of 8-week-old mice and revealed a further increase in islet preparations of normoglycemic 13- to 14-week-old mice (Fig. 1A). The level of expression of miR-29a/b/c in lymphocytes escaping the islets of NOD mice was comparable to the level detected in the islets of 4-week-old mice (Fig. 1B). Moreover, islets of 14-week-old mice incubated for 24 h to permit the escape of infiltrating lymphocytes retained strongly elevated levels of miR-29a/b/c (Fig. 1B). In agreement with these observations, in situ hybridization of NOD pancreas at different ages, combined with an immunofluorescence staining for insulin on close by sections, confirmed that the bulk of miR-29a (Supplementary Fig. 4, *lower panels*, and Supplementary Fig. 5) and miR-29b (not shown) is concentrated in insulin-producing cells and not in infiltrating immune cells.

Taken together, these findings indicate that the rise of miR-29a/b/c reflects changes occurring in β -cells and is not merely the result of the presence of immune cells in the islets. The observed increase in miR-29 expression is also not the result of mice aging. Indeed, miR-29a/b/c levels

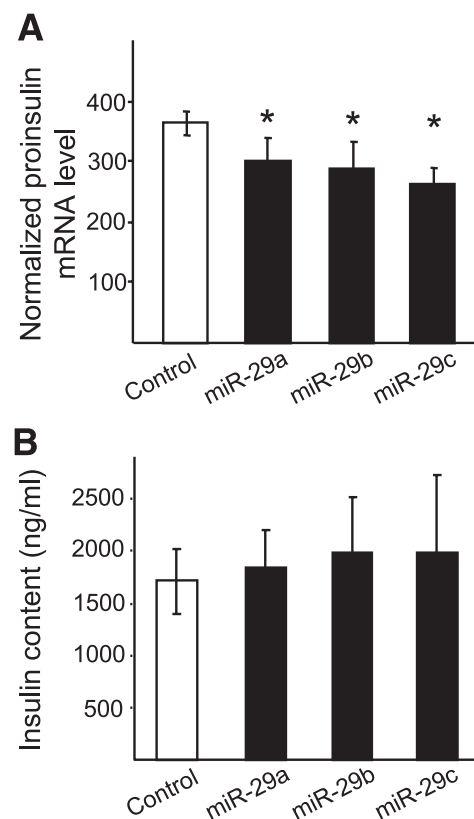


FIG. 3. miR-29 overexpression does not change insulin content. **A:** MIN6 were transfected with a control RNA duplex or with duplexes corresponding to the mature forms of miR-29a, miR-29b, and miR-29c. Proinsulin mRNA levels were measured 2 days later by qPCR. Results are expressed as the ratios between proinsulin and 18S mRNA and shown as mean \pm SD of four independent experiments. **P* < 0.05 vs. controls. **B:** Under these conditions, the insulin content of MIN6 cells, as determined by enzyme-linked immunosorbent assay, was not significantly altered. The data shown represent the mean \pm SD of seven independent experiments.

were not significantly different between the islets of 6- and 12-week-old C57BL/6 mice (data not shown), and no significant changes were observed in the islets of 13-week-old NOD-SCID mice that do not develop type 1 diabetes. In contrast, proinsulin 2-deficient mice, which display accelerated insulinitis and diabetes (34), showed a dramatic rise in miR-29a/b/c expression already at age 8 weeks (Supplementary Fig. 6).

Incubation of MIN6 cells (Fig. 2A), mouse islets (Fig. 2B), or dissociated human islet cells (Fig. 2C) in the presence of $\text{IL-1}\beta$ (0.1 ng/mL; 50 units/mL), $\text{TNF-}\alpha$ (10 ng/mL; 500 unit/mL), and $\text{IFN-}\gamma$ (30 ng/mL; 50 units/mL) led to a two-fold increase in the expression of the three miR-29 family members, suggesting that at least part of the observed induction occurring during early stages of diabetes development in NOD mice was linked to the chronic exposure of β -cells to these proinflammatory cytokines.

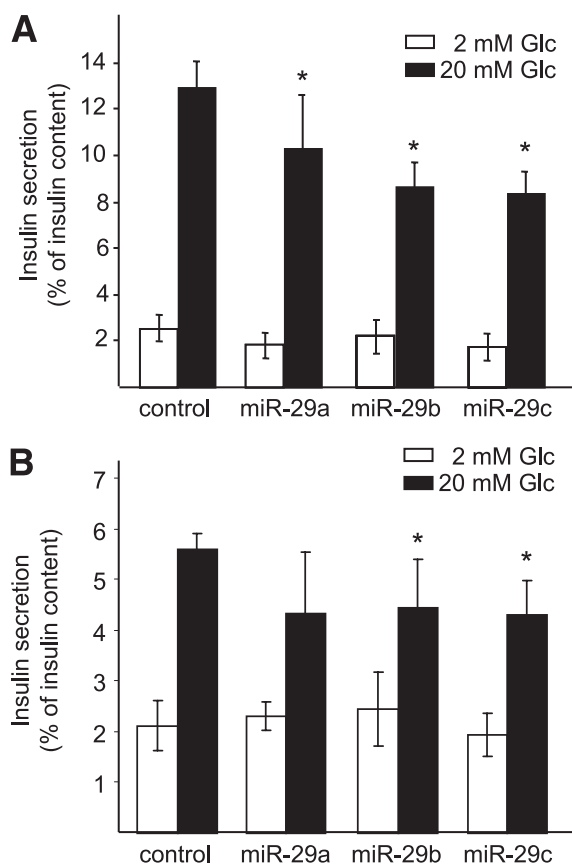


FIG. 4. miR-29 overexpression alters glucose-induced insulin secretion. **A:** MIN6 cells were transiently transfected with a control RNA duplex or with duplexes corresponding to the mature forms of miR-29a, miR-29b, and miR-29c. Two days later, the cells were incubated in the presence of 2 or 20 mmol/L glucose (Glc) for 45 min. The amount of insulin secreted during the incubation period was assessed by enzyme-linked immunosorbent assay (ELISA). Values were expressed as percentage of insulin content and are shown as mean \pm SD of five independent experiments. **B:** Cells dispersed from mouse pancreatic islets were plated and transfected with a control small interfering RNA or with the indicated oligonucleotide duplexes. Three days later, the cells were incubated in the presence of 2 or 20 mmol/L glucose for 45 min. The amount of insulin secreted during the incubation period was assessed by ELISA. Values were expressed as the percentage of insulin content and are shown as the mean \pm SD of three independent experiments. Total insulin content was 16 ± 4 , 18 ± 5 , 16 ± 4 , and 16 ± 5 for control, miR-29a, b, and c, respectively. * $P < 0.05$ vs. control cells stimulated with 20 mmol/L glucose.

To test the effect of miR-29a/b/c overexpression on pancreatic β -cell functions, oligonucleotides mimicking the mature sequence of each of the miR-29 family members were transiently transfected into MIN6 cells. This transfection resulted in a cellular content of the respective miRNA, which was comparable to that observed in the islets of 8-week-old NOD mice (Supplementary Fig. 7). We first assessed the effect of alterations in the level of the three miRNAs on insulin biosynthesis. Overexpression of miR-29 family members led to a small decrease in proinsulin mRNA levels but did not significantly modify total insulin content (Fig. 3). We then investigated the effects on insulin release. The rise of miR-29a/b/c in MIN6 cells did not affect basal insulin secretion (Fig. 4A). In contrast, glucose-induced insulin release was decreased by 20–40%. A similar reduction in insulin secretion elicited in the presence of glucose was detected upon overexpression of miR-29 family members in primary mouse islet cells (Fig. 4B) and in primary human islet cells (Supplementary Fig. 8).

The transcription factor *Onecut2* is a predicted target of miR-29a/b/c (<http://www.targetscan.org>) (35). We previously demonstrated that *Onecut2* modulates the expression of granuphilin, a secretory granule protein acting as a potent inhibitor of insulin exocytosis (8) that is expressed in all β -cells of nondamaged islets, irrespective of the age of NOD mice (data not shown). In agreement with the computational predictions, transfections under the conditions that led to miR-29a/b/c overexpression resulted in a decrease in the level of *Onecut2* and a consequent rise in the expression of granuphilin (Fig. 5) in MIN6 cells and in dissociated mouse islet cells, potentially explaining at least part of the effect of miR-29 family members on glucose-induced insulin release. In contrast, we were unable to detect significant changes in the level of *Syntaxin 1a*, a key component of the machinery of insulin exocytosis (36,37), which is also identified by computational programs as a potential target of miR-29 (Fig. 5).

The effect of elevated levels of miR-29 family members on cell survival was next investigated. Overexpression of miR-29a/b/c in MIN6 cells (Fig. 6A, left panel) and in mouse (Fig. 6B, left panel) and human dissociated islet cells (Fig. 6C) led to a significant increase in cell death, as assessed by scoring the cells displaying pyknotic nuclei.

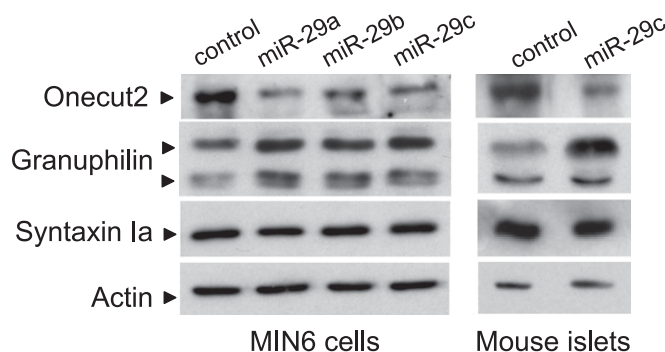


FIG. 5. Overexpression of miR-29 selectively alters the expression of proteins involved in insulin exocytosis. MIN6 cells (left panel) or dissociated mouse islet cells (right panel) were transiently transfected with RNA duplexes that allow for overexpression of miR-29a, b, or c. Western blotting showed that this change was associated to a reduction in the levels of *Onecut2* and to an increase in the levels of granuphilin, without alterations of *Syntaxin 1a*. A representative of three to five experiments is shown.

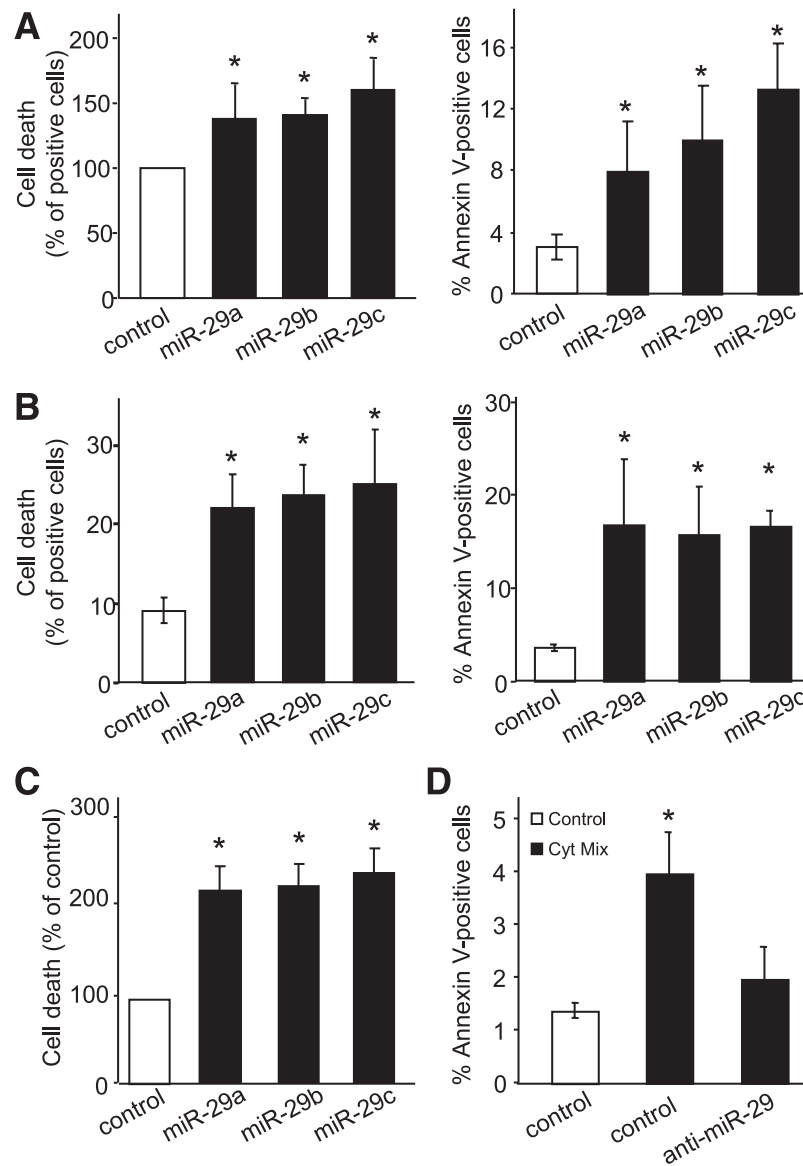


FIG. 6. Overexpression of miR-29 increases the apoptosis of transformed and primary β -cells. **A:** MIN6 cells were transiently transfected with a control RNA duplex or with duplexes leading to miR-29a, miR-29b, and miR-29c overexpression. Three days later, the monolayers were stained with Hoechst dye 33342 to score for dead cells (*left panel*) or with Annexin V-enhanced green fluorescent protein (EGFP) to score for apoptotic cells (*right panel*). **B:** Islet cells were dispersed from mouse islets and plated. Adherent cells were transfected with the indicated duplexes. Three days later, the monolayers were stained with Hoechst dye 33342 (*left panel*) or with Annexin V-EGFP (*right panel*). **C:** Dissociated human islet cells were transfected with a control RNA duplex or with duplexes leading to miR-29a, miR-29b, and miR-29c overexpression. Three days later, the monolayers were stained with Hoechst dye 33342 to score for cells displaying pyknotic nuclei. **D:** MIN6 cells were transfected with a control oligonucleotide or with a modified antisense oligonucleotide that specifically inhibits miR-29c (anti-miR-29c). The day after, the cells were incubated with or without IL-1 β (0.1 ng/mL) plus TNF- α (10 ng/mL) and IFN- γ (30 ng/mL), and 24 h later, the glass coverslips cells were stained with Annexin V-EGFP to score the fraction of apoptotic cells. All figures show the mean \pm SD of three (*B* and *C*) to five (*A*) independent experiments. * $P < 0.05$ vs. control.

Identical results were obtained when apoptosis was assessed by Annexin V labeling (Fig. 6A and B, *right panels*). In contrast, the number of necrotic cells determined by propidium iodide labeling remained unchanged (data not shown). Pretreatment with anti-miR-29c significantly decreased, to control levels, the number of MIN6 cells undergoing apoptosis in the presence of IL-1 β , TNF- α , and IFN- γ (Fig. 6D).

We then attempted to elucidate the mechanisms through which a rise in the level of miR-29a/b/c can trigger β -cell death. Interestingly, Mcl1, an antiapoptotic protein belonging to the Bcl2 family and playing a key role in β -cell survival (38), is a predicted target of miR-29a/b/c (26,39).

Indeed, we found that increased levels of miR-29a/b/c inhibited the expression by MIN6 cells of a luciferase construct containing the 3'UTR sequence of Mcl1 (Fig. 7A). This effect was abrogated by mutation of the sequence recognized by these miRNAs, indicating a direct interaction with the 3'UTR of Mcl1. Western blot analysis confirmed a significant reduction of the level of Mcl1 in MIN6 cells and in mouse islet cells overexpressing the miR-29 family members (Fig. 7B and C). No significant changes in MCL1 mRNA levels were detected in miR-29 overexpressing cells, indicating that the effect of the miRNAs is mainly post-transcriptional (Supplementary Fig. 9).

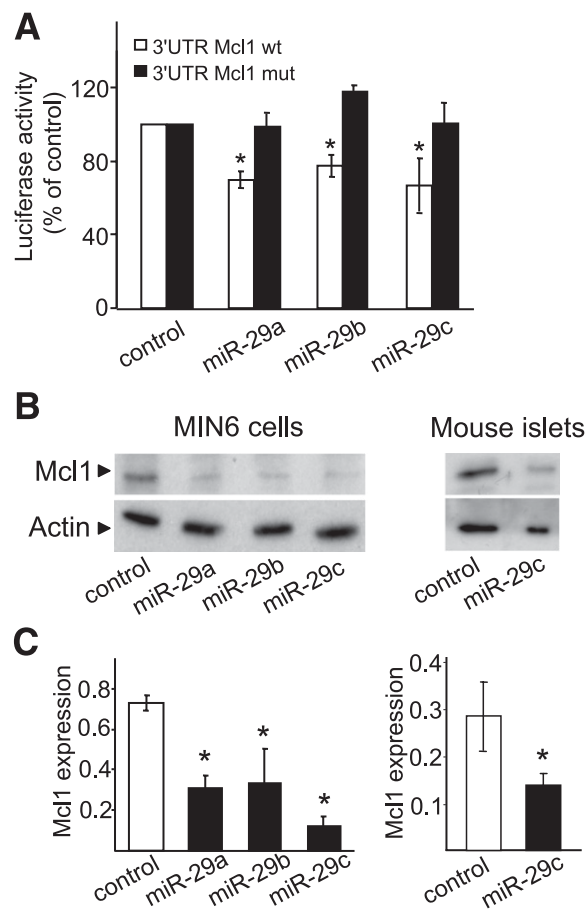


FIG. 7. Mcl1 is a direct target of miR-29 family members. **A:** MIN6 cells were cotransfected with a *Renilla* luciferase construct and *Firefly* luciferase reporter plasmids containing the wild-type (wt) or a mutated (mut) sequence of the 3'UTR of mouse Mcl1 and RNA duplexes, leading to miR-29a, miR-29b, or miR-29c overexpression. Luciferase activities were measured 3 days later. *Firefly* luciferase activities were divided by the *Renilla* luciferase activities to correct for differences in transfection efficiency. The value obtained under control condition was set to 100%. Values are mean \pm SD of four independent experiments. **B:** MIN6 cells (*left panel*) or dissociated mouse islet cells (*right panel*) were transfected with the indicated duplexes. Expression of Mcl1 and actin was assessed by Western blotting. A representative of three experiments is shown. **C:** Quantification of the effect of miR-29a/b/c overexpression on Mcl1 expression by densitometric scanning of the films in MIN6 cells (*left panel*) and mouse islets (*right panel*). The results are expressed as means \pm SD of three independent experiments. * $P < 0.05$ vs. control.

To demonstrate a direct link between the reduction of Mcl1 and the effect of miR-29 on β -cell apoptosis, MIN6 cells were transfected with an oligonucleotide designed to specifically protect the Mcl1 mRNA from miR-29 binding, without interfering with the regulation of other miR-29 targets (25). The reduction in Mcl1 levels elicited by miR-29c overexpression or by proinflammatory cytokines was efficiently prevented by transfection of this target protector (Supplementary Fig. 10A and B). As shown in Fig. 8A, the cells expressing the Mcl1 target protector were resistant to apoptosis induced by miR-29 overexpression. Moreover, the cells transfected with the Mcl1 target protector were not sensitive to cytokine-induced apoptosis (Fig. 8B), indicating that at least part of the deleterious effect of these inflammatory mediators on β -cell survival is caused by a miR-29-induced drop in the expression of the antiapoptotic Mcl1 protein.

DISCUSSION

During the initial phases of type 1 diabetes, pancreatic β -cells are chronically exposed to inflammatory mediators released by immune cells invading the islets of Langerhans. This results in major changes in gene expression, with

consequent defects in the accomplishment of specialized functions, and loss of the cells by apoptosis (1). We recently demonstrated that part of the changes in gene expression triggered by proinflammatory cytokines reflects alterations in the level of a new class of regulatory molecules, the miRNAs (11). Indeed, we found that prolonged exposure to IL-1 β or TNF- α results in a strong upregulation of miR-21, miR-34a, and miR-146a, with large effects on the secretory capacities of β -cells and their sensitivity to apoptosis (11). In this study, we confirm these findings and further extend them via the identification of additional miRNAs, whose expression level is markedly altered with the onset of type 1 diabetes in NOD mice. The members of the miR-29 family, which are among the most abundant miRNAs expressed in mouse pancreatic β -cell (11,30), increased with the age of prediabetic but still normoglycemic NOD mice. In situ hybridization and qPCR analysis, coupled with immunofluorescence for insulin, demonstrated that the rise in miR-29 expression occurred in β -cells and not in invading immune cells.

The mechanism responsible for this induction of miR-29a/b/c in NOD mice remains to be fully elucidated. We found that exposure of MIN6 cells and human islets to proinflammatory cytokines causes a twofold increase in

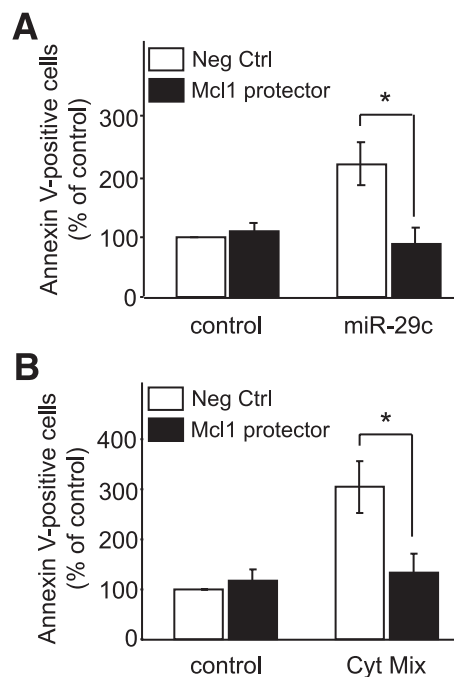


FIG. 8. miR-29- and cytokine-induced apoptosis is blocked by preventing binding to Mcl1. **A:** MIN6 cells were transfected with a control RNA duplex or with duplexes corresponding to the mature form of miR-29c, together with a scrambled miScript Target Protector as a negative control or a Mcl1 miScript Target Protector. Three days after transfection, the cells were stained with Annexin V-enhanced green fluorescent protein (EGFP) to score the fraction of apoptotic cells. **B:** MIN6 cells were transfected with the scrambled miScript Target Protector as a negative control or with the Mcl1 miScript Target Protector. Two days later, the cells were treated for 24 h with a mix of cytokines (Cyt Mix) including IL-1 β (0.1 ng/mL), TNF- α (10 ng/mL), and IFN- γ (30 ng/mL). At the end of the incubation, the cells were stained with Annexin V-EGFP to score the fraction of apoptotic cells. All figures show the mean \pm SD of three independent experiments. * $P < 0.05$ vs. control.

the expression of miR-29 family members. These results are in good agreement with those obtained previously by microarray analyses (11). The signaling pathways involved in the induction of miR-29 family members have not been dissected yet. Zhou et al. (40) have recently identified in the promoter of miR-29a potential binding sites for NF κ B, a transcription factor known to contribute to cytokine-mediated β -cell dysfunction and to the development of type 1 diabetes (41). Expression of miR-29 was also reported to be modulated by p53 (42), a transcription factor that is induced in response to proinflammatory cytokines (43) and that we have shown controls the expression of other miRNAs (7). Additional cytokines, such as IL-4 and TGF- β , which are defectively produced in NOD mice (44–46), have been reported to inhibit miR-29 expression in other cell systems (47). A deficit of these cytokines may potentially contribute to the strong upregulation of miR-29a/b/c that is observed in the islets of prediabetic NOD mice.

Our results demonstrate that miR-29 overexpression downregulates glucose-induced insulin secretion of primary mouse and human islet cells as well as of MIN6 cells. This downregulation is associated with the interaction of the miRNAs with Onecut2, one of their putative targets. This transcription factor controls the expression of granuphilin/Syt1-4 (8), a Rab guanine triphosphate (GTP)ase effector that colocalizes with the dense-core, insulin-containing granules of pancreatic β -cells and acts as a potent inhibitor of insulin exocytosis (23,48). Thus, by decreasing Onecut2 levels, miR-29 enables the expression of increased amounts of granuphilin, resulting in impaired stimulation of insulin secretion. Consistent with this mechanism, we found that granuphilin is abundant in most β -cells, irrespective of the

age of the NOD mice, and the presence of peri- or intraislet insulinitis.

Prolonged exposure to proinflammatory cytokines is known to affect β -cell survival. Overexpression of miR-29a, -b, or -c to levels comparable to those observed in 8-week-old NOD mice mimics the effect of the cytokines. Moreover, blockade of miR-29 protects β -cells from the deleterious effect of these inflammatory mediators. In line with results obtained in other cell types (26,39,49), Western blot analysis and luciferase reporter assays demonstrated a decrease in the level of the antiapoptotic protein Mcl1 on miR-29 overexpression. Downregulation of this antiapoptotic protein is a crucial event, leading to β -cell apoptosis in response to cytokines (38). Our data are in good agreement with these observations and suggest that, as it is the case in other cell systems, even a modest reduction of Mcl1 levels caused by miR-29 overexpression can result in loss of mitochondrial potential, cytochrome c release, and activation of caspase-3 (39). Indeed, an oligonucleotide specifically designed to mask the binding site of miR-29 on Mcl1 3'UTR, without affecting the stability or the translation of the mRNA (25), restored the expression of the antiapoptotic protein, and prevented both miR-29- and cytokine-mediated apoptosis. Our findings suggest that molecules capable of protecting individual targets represent attractive tools to precisely dissect the mode of action of miRNAs and could also serve as therapeutic strategies to selectively promote the expression of single genes beneficial for β -cells.

The miR-29 family members have been shown to contribute to insulin resistance in insulin target tissues of GK rats (33). Interestingly, type 1 diabetes in NOD mice is associated with insulin resistance (50). Moreover, miR-29

has recently been reported to inhibit innate and adaptive immune responses by suppressing the production of IFN- γ in natural killer cells and T lymphocytes (51). Thus, changes in miR-29 expression may play a broader role in the pathogenesis of type 1 diabetes.

The current study was carried out in NOD mice, an animal model sharing many characteristics of type 1 diabetes in humans and that has significantly contributed to our understanding of the molecular processes associated with the development of this disease (52). It will now be important to confirm that the mechanisms identified in NOD mice and β -cell lines are indeed also operating during the initial phases of type 1 diabetes in humans. Should this turn out to be the case, a better knowledge of islet miRNA dysfunction may open the way to alternative strategies to delay and prevent the development of the disease in newly diagnosed type 1 diabetic patients.

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E.R. researched data, contributed to discussion, and wrote the manuscript. S.G. and D.C. researched data. C.Br. researched data and contributed to discussion. C.Bo. contributed to discussion. P.M. researched data, contributed to discussion, and reviewed and edited the manuscript. R.R. contributed to discussion, and wrote, reviewed, and edited the manuscript. R.R. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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