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ARTICLE

Identification of particular groups of microRNAs that positively or negatively impact on beta cell function in obese models of type 2 diabetes

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Abstract
Aims/hypothesis MicroRNAs are key regulators of gene expression involved in health and disease. The goal of our study was to investigate the global changes in beta cell microRNA expression occurring in two models of obesity-associated type 2 diabetes and to assess their potential contribution to the development of the disease.
Methods MicroRNA profiling of pancreatic islets isolated from prediabetic and diabetic *db*/*db* mice and from mice fed a high-fat diet was performed by microarray. The functional impact of the changes in microRNA expression was assessed by reproducing them in vitro in primary rat and human beta cells.
Results MicroRNAs differentially expressed in both models of obesity-associated type 2 diabetes fall into two distinct categories. A group including miR-132, miR-184 and miR-338-3p displays expression changes occurring long before the onset of diabetes. Functional studies indicate that these expression changes have positive effects on beta cell activities and mass. In contrast, modifications in the levels of miR-34a, miR-146a, miR-199a-3p, miR-203, miR-210 and miR-383 primarily occur in diabetic mice and result in increased beta cell apoptosis. These results indicate that obesity and insulin resistance trigger adaptations in the levels of particular microRNAs to allow sustained beta cell function, and that additional microRNA deregulation negatively impacting on insulin-secreting cells may cause beta cell demise and diabetes manifestation.
Conclusions/interpretation We propose that maintenance of blood glucose homeostasis or progression toward glucose intolerance and type 2 diabetes may be determined by the balance between expression changes of particular microRNAs.

Keywords Apoptosis · Beta cell · Diabetes · High-fat diet · Insulin resistance · MicroRNA · Pancreatic islet · Obesity · Secretion

Abbreviations

cMET	Met proto-oncogene (hepatocyte growth factor receptor)
GSK-3β	Glycogen synthase kinase 3 β
HFD	High-fat diet
miRNA	microRNA
mTOR	Mammalian target of rapamycin
PRL	Prolactin
qRT-PCT	Quantitative RT-PCR

Introduction

Type 2 diabetes is characterised by insulin resistance of target tissues and insufficient insulin secretion from pancreatic beta cells to meet the organism's needs. Insulin resistance is normally compensated by expansion of the beta cell mass and a rise in the insulin secretory activity [1]. However,

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in predisposed individuals this compensatory process fails, resulting in beta cell dysfunction, eventually accompanied by reduction of the beta cell mass and type 2 diabetes manifestation [2]. A better knowledge of the molecular mechanisms underlying beta cell adaptation and failure will be instrumental for designing new strategies to prevent or treat this disease.

MicroRNAs (miRNAs) are small non-coding RNAs that play central roles in a number of physiological and pathological processes [3]. Several studies have shown that miRNAs participate in the control of beta cell differentiation, function and mass. These non-coding RNAs regulate insulin production by directly or indirectly affecting the expression of key transcription factors and they contribute to fine-tuning of hormone release by modulating the levels of important components of the beta cell secretory machinery [4]. The expression of several miRNAs is affected by prolonged exposure to elevated concentrations of glucose, NEFA and proinflammatory cytokines [4]. Moreover, alterations in the levels of many islet miRNAs have been reported in different models of diabetes [5–9]. However, the functional impact of these miRNA expression changes and their potential role in the development of diabetes were, in most cases, not explored.

In this study, we analysed the global variations in islet miRNA expression in prediabetic and diabetic *db*/*db* mice [10] and in mice fed a high-fat diet (HFD) [11]. Differentially expressed miRNAs in these models of obesity-associated diabetes were systematically investigated for their effects on rat and human beta cell function and for their impact on cell survival on chronic exposure to pro-apoptotic conditions. The results indicate that specific changes in islet miRNA expression in prediabetic and diabetic states reflect the coexistence of adaptive processes elicited to compensate insulin resistance and of pathological reactions promoting beta cell failure. The balance between these opposing phenomena is likely to determine progression from normoglycaemia to hyperglycaemic states and the manifestation of diabetes.

Methods

Materials TNFα and INFγ were obtained from R&D Systems (Minneapolis, MN, USA). IL-1β, prolactin (PRL), exendin-4 and palmitate were purchased from Sigma-Aldrich (St Louis, MO, USA).

Animals Prediabetic (6 weeks old) and diabetic (14–20 weeks old) C57BL/KsJ *db*/*db* mice and age-matched C57BL/KsJ control animals were obtained from the Garvan Institute breeding colonies (Sydney, NSW, Australia) [10]. Five-week-old male C57BL/6 mice were purchased from Charles River Laboratories (Saint-Constant, QC, Canada) and fed a normal diet or HFD (Bio-Ser Diet number F3282, Frenchtown, NJ, USA; 60% [wt/wt] energy from fat) for 8 weeks as described [11]. Male Wistar rats were purchased from Charles River Laboratories (L'Arbresle, France). All animal procedures were performed in accordance with National Institutes of Health (NIH) guidelines and were approved by the respective Australian, Canadian and Swiss research councils and veterinary offices.

Microarray profiling Total RNA was isolated with the mirVana RNA isolation kit (Ambion, Austin, TX, USA) from islets of C57BL/KsJ *db*/*db* mice or control animals. Total RNA from islets of C57BL/6 mice fed a normal diet or HFD was isolated with the miRNeasy kit (Qiagen, Hombrechtikon, Switzerland). Global miRNA expression profiling was carried out at the Genomic Technologies Facility of the University of Lausanne using miRNA gene microarrays (Agilent Technologies, Morges, Switzerland). Microarrays included probes for mouse miRNAs listed on www.mirbase.org/ (release 14, 2009).

Isolation and culture of dissociated islet cells Pancreatic islets were isolated as described previously [12] by collagenase digestion followed by purification on a Histopaque (Sigma-Aldrich) density gradient. The islets were first cultured overnight in RPMI 1640 Glutamax medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (vol./vol.) FCS (Amimed, BioConcept, Allschwill, Switzerland), 50 U/ml penicillin, 50 μg/ml streptomycin, 1 mmol/l Na pyruvate and 250 μmol/l HEPES, and then dissociated by incubation with trypsin (5 mg/ml at 37°C for 4–5 min). Human pancreatic islets were obtained from the Cell Isolation and Transplantation Center (University of Geneva), through the ECIT 'Islets for Research' distribution programme sponsored by the JDRF. The use of human islets was approved by the Geneva institutional Ethics Committee. Dissociated human islet cells prepared using the procedure described above were cultured in CMRL medium (Invitrogen) supplemented with 10% (vol./vol.) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l glutamine and 250 μmol/l HEPES. Detailed information about the human islet preparations used in this study is presented in electronic supplementary material (ESM) Table 1.

MIN6B1 cell culture The murine insulin-secreting cell line MIN6B1 [13] was cultured at a density of 1.5×10^5 cells/cm^2 in DMEM-Glutamax medium (Invitrogen) supplemented with 15% (vol./vol.) FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 70 μmol/l β-mercaptoethanol.

Transfection and modulation of miRNA levels MIN6B1 and dissociated rat or human islet cells were transfected with Lipofectamine 2000 (Invitrogen) with RNA oligonucleotide duplexes (Eurogentec, Seraing, Belgium) corresponding to the mature miRNA sequence (overexpression) or with single-stranded miScript miRNA inhibitors (Qiagen, Hombrechtikon, Switzerland) that specifically block endogenous miRNAs [14].

A custom-designed small interfering (si)RNA duplex directed against green fluorescent protein (sense 5′-GACGUAAACG GCCACAAGUUC-3′ and antisense 5′-ACUUGUGGCCGU UUACGU CGC-3′) and the miScript miRNA reference inhibitor (Qiagen) were used as negative controls for miRNA overexpression and downregulation, respectively.

Measurement of miRNA and mRNA expression Mature miRNA expression was assessed with the miRCURY LNA™ Universal RT MicroRNA PCR kit (Exiqon, Vedbaek, Denmark). Measurement of the levels of putative target mRNAs was performed by conventional reverse transcription (Promega, Dübendorf, Switzerland) followed by quantitative RT-PCR (qRT-PCR; Biorad, Reinach, Switzerland) with custom-designed primers (Microsynth, Balgach, Switzerland), details of which are available on request. MiRNA expression was normalised to the level of U6 or miR-7 (an islet-specific miRNA used as internal control) while mRNA expression was normalised to 18S.

Insulin secretion At 2 days after transfection, MIN6B1 or dissociated rat islet cells were pre-incubated for 30 min at 37°C in Krebs buffer (127 mmol/l NaCl, 4.7 mmol/l KCl, 1 mmol/l $CaCl_2$, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 5 mmol/l $NaHCO_3$, 0.1% [wt/vol.] BSA, 25 mmol/l HEPES, pH 7.4) containing 2 mmol/l glucose. The pre-incubation medium was discarded and the cells incubated for 45 min in the same buffer (basal conditions). After collecting the supernatant fractions, the cells were incubated for 45 min in Krebs buffer containing 20 mmol/l glucose (stimulatory conditions). The incubation medium was collected and total cellular insulin contents recovered in acidified ethanol (75% [vol./vol.] ethanol, 0.55% [vol./vol.] HCl). The amount of insulin in the samples was determined using an insulin enzyme immunoassay kit (SPI-Bio, Bertin Pharma, Montigny le Bretonneux, France).

Cell death assessment Transfected MIN6B1, rat or human dissociated islet cells were incubated with 1 μg/ml Hoechst 33342 (Invitrogen) for 1 min. The fractions of cells (at least 1×10^3 per condition) displaying pycnotic nuclei were scored under fluorescence microscopy (AxioCam MRc5; Zeiss, Feldbach, Switzerland). Apoptosis was triggered by exposing the cells to cytokines (30 ng/ml INFγ, 10 ng/ml TNFα and 0.1 ng/ml IL-1β) for 24 h or to medium (5% [vol./vol.] FCS) supplemented with 0.5 mmol/l palmitate bound to 0.5% [wt/vol.] BSA [15] for 48 h.

Proliferation assay Transfected MIN6B1 or dissociated islet cells cultured on poly-L-lysine-coated glass cover slips were fixed with ice-cold methanol and permeabilised with 0.5% (wt/vol.) saponin (Sigma-Aldrich, St Louis, MO, USA). The cover slips were incubated with antibodies against Ki67 (1:500) (Abcam, Cambridge, UK) and insulin (1:500) (Millipore, Zug, Switzerland) and then with anti-rabbit Alexa-Fluor-488 and anti-mouse Alexa-Fluor-555 antibodies (Invitrogen). At the end of the incubation, the cover slips were washed with PBS containing Hoechst 33342 (Invitrogen) and images of at least 1×10^3 cells per condition were collected using a fluorescence microscope. PRL (500 ng/ml for 48 h) was used as positive control.

Protein extraction and western blotting Protein lysates (30–50 μg) from MIN6B1 cells prepared as described previously [9] were separated on polyacrylamide gels and transferred to polyvinylidine fluoride membranes. The membranes were incubated overnight with antibodies against granuphilin [16] (1:2,000); mammalian target of rapamycin (mTOR; 2972, 1:1,000 Cell Signaling, Danvers, MA, USA), met proto-oncogene (hepatocyte growth factor receptor) (cMET; Cell Signaling, 3127, 1:1,000) and glycogen synthase kinase 3β (GSK-3β; Cell Signaling 9315, 1:1,000). Antibodies against α-tubulin (T9026, 1:10,000, Sigma-Aldrich) and actin (Clone C4 MAB1501, 1:15,000, Millipore) were used to verify equal loading. After exposure to IRDye (Li-Cor Biosciences, Bad Homburg, Germany) or horseradish-peroxidase-coupled secondary antibodies for 1 h, the bands were visualised via the Odyssey imaging system (Li-Cor Biosciences) and chemiluminescence (GE Healthcare Europe, Glattbrugg, Switzerland), respectively. Band intensity was quantified by ImageJ software.

Statistical analysis Statistical differences were assessed using a Student's *t* test or, for multiple comparisons, one-way ANOVA of the means, followed by a post-hoc Dunnett test (SAS statistical package; SAS, Carry, NC, USA).

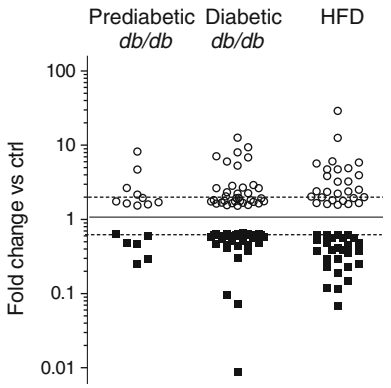

Fig. 1 miRNAs differentially expressed in pancreatic islets of animal models of type 2 diabetes. Pancreatic islets were isolated from prediabetic (6 weeks old) and diabetic (14–20 weeks old) *db*/*db* mice and from HFD-fed mice. miRNA expression levels were analysed by microarray. Data are expressed as fold changes vs the corresponding controls and are presented on a logarithmic scale ($\log_{10}$). Only miRNAs displaying increases (white circles) or decreases (black squares) of at least 1.5 fold ($p\leq0.05$) are plotted. Dashed lines correspond to a twofold change. Ctrl, control

Results

Islet miRNA expression in rodent models of type 2 diabetes To investigate the contribution of miRNAs to beta cell dysfunction and the development of type 2 diabetes, we performed global miRNA expression profiling in pancreatic islets obtained from: *db*/*db* mice, which lack the leptin receptor and develop severe obesity associated with type 2 diabetes [10, 17]; and diet-induced obese mice, which display mild hyperglycaemia and beta cell dysfunction after being fed an HFD for 8 weeks [11]. The characteristics of the animals used in this study are presented in ESM Tables 2–4. We identified more than 60 differentially expressed miRNAs in *db*/*db* and HFD-fed mice islets compared with their respective controls, with overlapping changes in the two models (Fig. 1).

For *db*/*db* mice, miRNA expression was determined in both prediabetic (6 weeks old) and diabetic (14–20 weeks old) animals. In prediabetic mice, the miRNAs displaying the most striking changes were miR-132, with expression increasing by 8.2-fold, and miR-210, miR-184 and miR-203, for which expression decreased by 4.0-, 3.4- and 2.0-fold, respectively (ESM Table 5). In agreement with our previous findings [9], the islets of prediabetic *db*/*db* mice contained lower levels of miR-338-3p. The reduction of miR-210 and miR-184 was more dramatic in the islets of overtly diabetic *db*/*db* mice (10.4- and 115-fold decrease, respectively), whereas upregulation of miR-132 and downregulation of miR-203 and miR-338-3p remained approximately constant in prediabetic and diabetic animals (ESM Tables 5 and 6). In addition to these changes, the islets of adult diabetic mice were characterised by alterations in the levels of additional miRNAs, including an upregulation of miR-199a-5p (12.6-fold) and miR-199a-3p (9.4-fold), a decline of miR-383 (13.7-fold) and, as previously reported [6], an increase of miR-34a

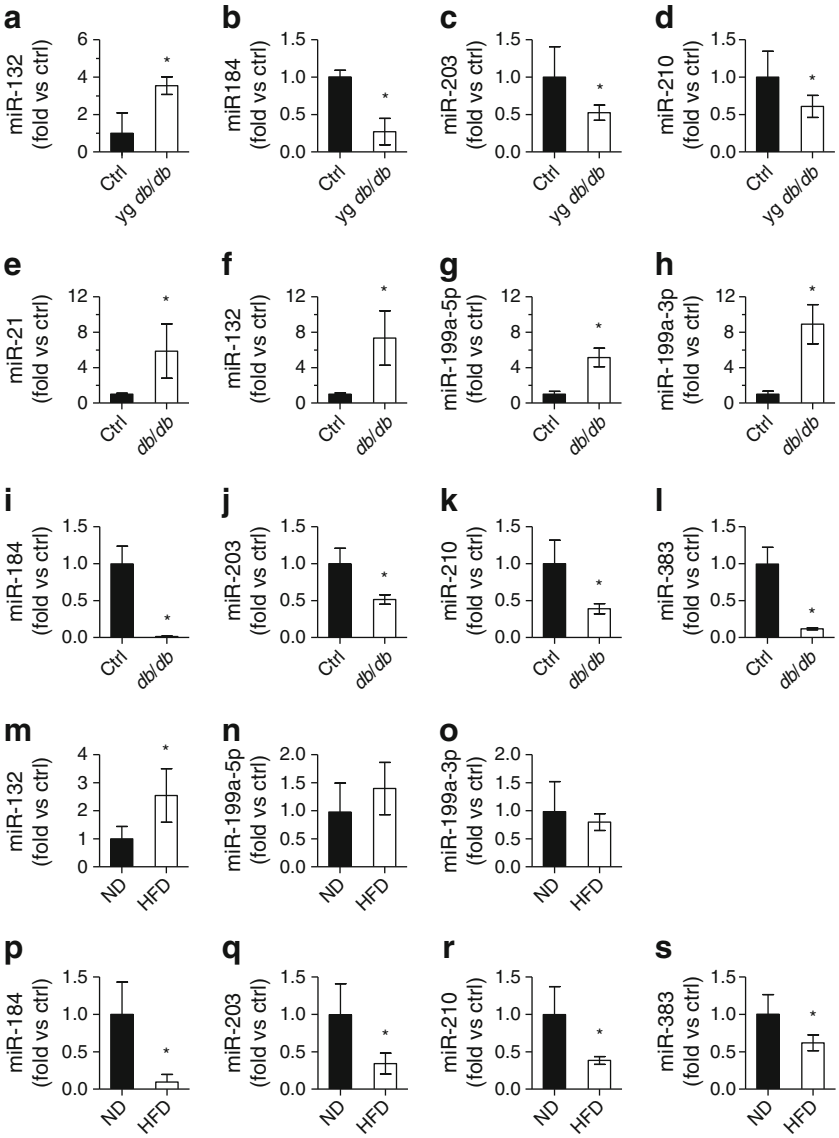

Fig. 2 Changes in miRNA expression in islets of type 2 diabetes animal models. The expression level of the indicated miRNAs was measured by qRT-PCR in pancreatic islets of young prediabetic (**a**–**d**) and diabetic (**e**–**l**) *db*/*db* mice vs age-matched control mice and in mice fed a normal diet or HFD (**m**–**s**). The results correspond to the mean ± SD of three to four animals per group and are normalised to the level of the respective controls. *Significantly different from control (control or normal diet, as indicated) ($p \leq 0.05$ by unpaired Student's *t* test). Ctrl, control; ND, normal diet; yg, young

and miR-146a (ESM Table 6). The results obtained by microarray analysis were confirmed by qRT-PCR (Fig. 2a–l). Our microarray data also revealed a 2.2-fold increase in miR-21, which we have previously shown to inhibit insulin secretion [8], a 1.6-fold decrease in miR-26a, which controls insulin biosynthesis [18], and an increase in miR-802 (sixfold), which regulates *Hnf1b* expression [19] (ESM Tables 6). The role of these miRNAs was not further investigated in this study.

Islet miRNA expression was also analysed in HFD-fed mice. For this purpose we selected the group of mice displaying the strongest response to HFD. These animals were markedly obese, insulin resistant, hyperinsulinaemic and clearly hyperglycaemic (ESM Table 4). HFD mice showed miRNA expression changes analogous to those observed in the islets of diabetic *db*/*db* mice, with the exception of miR-21, miR-34a, miR-146a, miR-199a-5p and miR-199a-3p, which were not significantly modified (ESM Table 7 and Fig. 2m–s).

Overall, the data indicate that a subset of islet miRNAs is similarly altered in two obesity-associated animal models of type 2 diabetes, suggesting a role of specific miRNAs in beta cell failure and the development of hyperglycaemia.

miRNA expression is affected by glucolipotoxic conditions To determine the possible causes of the changes in miRNA expression detected in the islets of *db*/*db* and HFD-fed mice, we tested whether the levels of these non-coding RNAs are affected by chronic exposure of beta cells to elevated concentrations of glucose and NEFA. We found that prolonged incubation of rat islets (Fig. 3) under glucolipotoxic conditions mimicked the modifications in miR-132, miR-184, miR-199a-3p, miR-203 and miR-383 expression observed in animal models. In contrast, under these glucolipotoxic conditions the levels of miR-210 and miR-199a-5p were not affected (Fig. 3).

Particular differentially expressed miRNAs influence beta cell functions and survival Modifications of miRNA expression in islets could reflect the activation of adaptive processes counterbalancing the increased insulin needs caused by obesity and insulin resistance or the onset of pathological conditions leading to beta cell dysfunction. Indeed, we have previously shown that downregulation of miR-338-3p contributes to compensatory beta cell mass expansion [9], whereas overexpression of miR-21, miR-34a and miR-146a negatively impacts on beta cell function [6, 8]. To assess the possible role of other differentially expressed miRNAs in these phenomena, we mimicked the changes observed in the animal models by transfecting dissociated rat islet cells and MIN6B1 cells with oligonucleotide duplexes corresponding to the mature miRNA sequences or with anti-miRNA molecules that specifically inhibit miRNAs (ESM Fig. 1). The transfected cells were then analysed for their functional properties.

We first assessed whether the miRNAs differentially expressed in type 2 diabetes models are involved in the regulation of insulin biosynthesis and release. Most of the studied miRNAs did not affect insulin content (Fig. 4a–c) or insulin release in dissociated rat islet cells (Fig. 4d–f) and MIN6B1 cells (ESM Fig. 2). However, overexpression of miR-132 resulted in improved glucose-stimulated insulin release from dissociated rat islet cells (Fig. 4d). In contrast, upregulation of miR-199a-5p led to an insulin secretory defect in MIN6B1 cells (ESM Fig. 2), but not in islet cells, where it only diminished the insulin content (Fig. 4a).

We next investigated whether the miRNAs differentially expressed in type 2 diabetes models regulate beta cell expansion. In MIN6B1 cells, upregulation of miR-132 or downregulation of miR-184, miR-203 and miR-383 led to an increase in proliferation while modification of the levels of other miRNAs had no significant effects (ESM Fig. 3). Proliferation of insulin-positive cells was also observed on

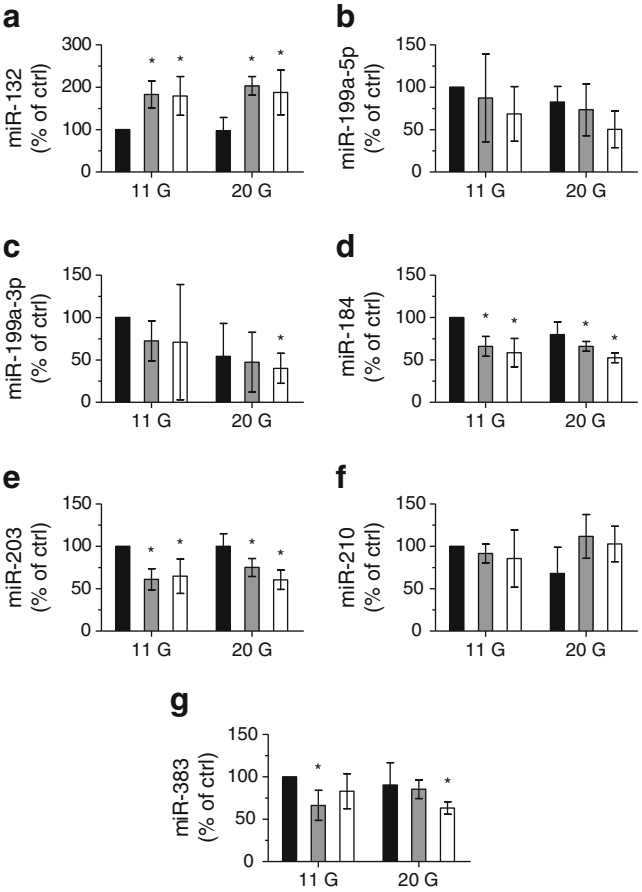

Fig. 3 Effect of chronically elevated glucose and palmitate on the level of islet miRNAs differentially expressed in type 2 diabetes animal models. Isolated rat islets were incubated at 11 or 20 mmol/l glucose with 0.5% BSA in the absence (black bars) or presence of 0.5 mmol/l palmitate for 48 h (grey bars) or 72 h (white bars). miRNA expression levels were measured by qRT-PCR, normalised by miR-7 and expressed as percentage of control (11 mmol/l glucose with 0.5% BSA) (**a**–**g**). *Significantly different from control condition, $p \leq 0.05$ by ANOVA analysis, Dunnett's post-hoc test. 11 G, 11 mmol/l glucose; 20 G, 20 mmol/l glucose; Ctrl, control

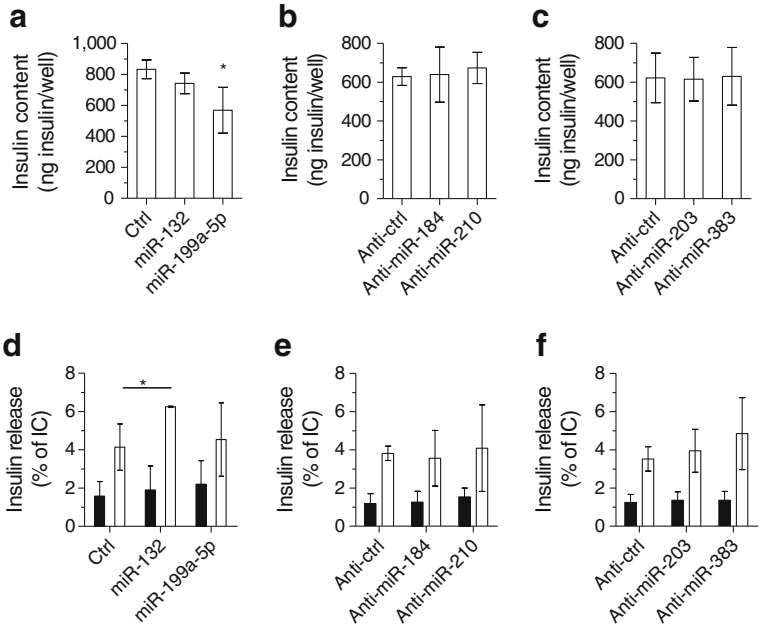

Fig. 4 Impact of changes in miRNA expression on insulin content and insulin secretion. Dispersed rat islet cells were transfected with oligonucleotides leading to overexpression (**a**, **d**) or downregulation (**b**, **c**, **e**, **f**) of the indicated miRNAs. Insulin content (**a**–**c**) and insulin secretion (**d**–**f**) in response to 2 (black bars) or 20 (white bars) mmol/l glucose were measured 48 h post-transfection. Insulin release is expressed as percentage of insulin content. *Significantly different from control condition (control or anti-control, as shown, incubated at the same glucose concentration), $p \leq 0.05$ by ANOVA analysis, Dunnett's post-hoc test. Ctrl, control; IC, insulin content

upregulation of miR-132 (Fig. 5a) and, to a lesser extent, downregulation of miR-184 in dispersed rat islet cells (Fig. 5b). In contrast, downregulation of miR-203 and miR-383 in primary cells had no effect (Fig. 5c, d). Similar to our previous work with miR-338-3p [9], these findings suggest that modification of the levels of miR-132 and miR-184 contributes to compensatory beta cell mass expansion elicited in response to insulin resistance.

As an increase in beta cell apoptosis and a reduction in beta cell mass are thought to play a role in the development of type 2 diabetes [20], we investigated the impact of miRNAs of interest on beta cell survival. As previously observed for miR-34a and miR-146a [6], upregulation of miR-199a-3p or reduction of miR-203, miR-210 and miR-383 expression increased the number of apoptotic MIN6B1 cells (ESM Fig. 4) as well as dispersed rat islet cells (Fig. 6a, c, e). Similar results were obtained using dissociated human islet cells (Fig. 6b, d, f). In contrast, overexpression of miR-132 or silencing of miR-184 did not induce beta cell death, but rather protected dispersed rat (Fig. 7a–d) and human (Fig. 7e–h) islet cells from apoptosis when the cells were chronically exposed to elevated concentrations of NEFA or to proinflammatory cytokines. Analogous results were also obtained in MIN6B1 cells (ESM Fig. 5).

Impact of particular miRNA changes on candidate target gene expression As described above, *db*/*db* mouse islets are characterised by a specific rise in the levels of miR-21, miR-34a, miR-146a, miR-199a-3p and -5p and a downregulation of miR-203, miR-210 and miR-383 that possibly result in beta cell dysfunction and death. We previously found that miR-34a affects beta cell survival by directly targeting the anti-apoptotic protein B cell CLL/lymphoma 2 (BCL2) [6]. Combining

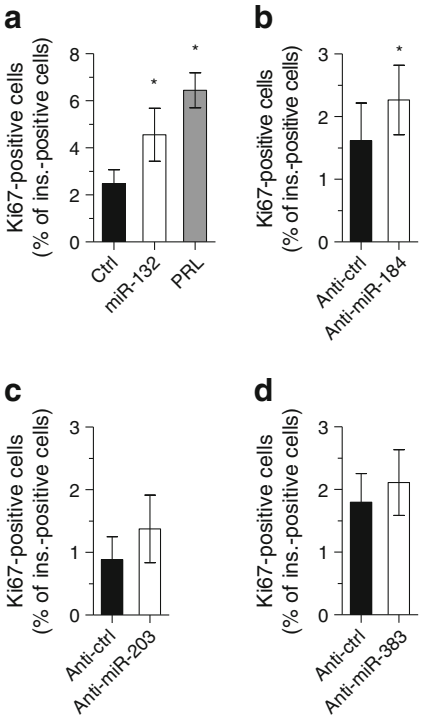

Fig. 5 Effect of specific modifications in miRNA expression on beta cell proliferation. Dispersed rat islet cells were transfected with oligonucleotides leading to overexperession (**a**) or downregulation (**b**–**d**) of the indicated miRNAs. Beta cell proliferation was assessed 72 h later by staining the cells with anti-Ki67 and anti-insulin antibodies. PRL (500 ng/ml for 48 h, grey bars) was used as positive control. The results correspond to the mean ± SD of three to six independent experiments. *Significantly different from control condition (control or anti-control, as shown), $p \leq 0.05$ by ANOVA analysis, Dunnett's post-hoc test. Ctrl, control; ins., insulin

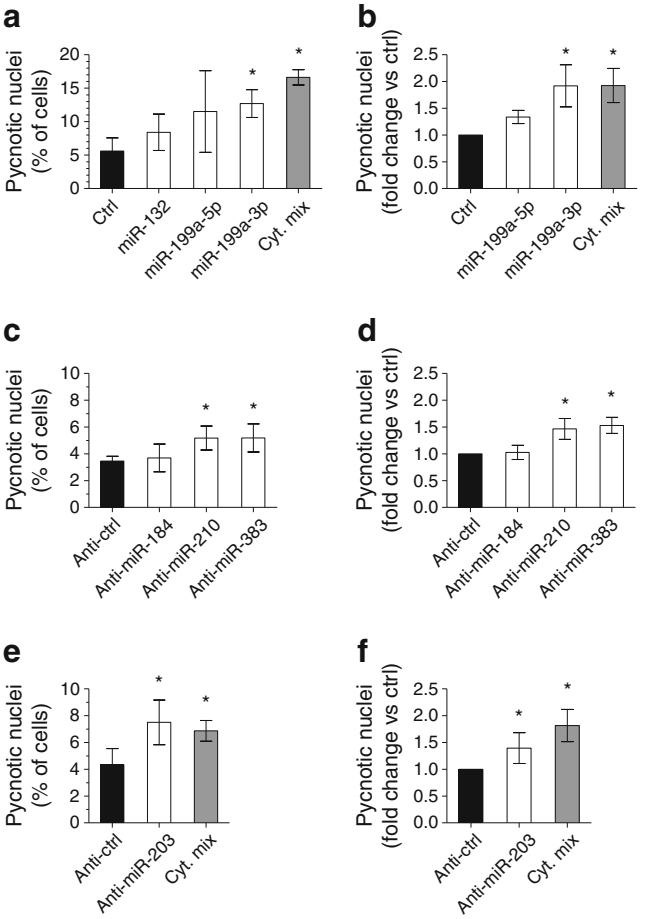

Fig. 6 Impact of specific miRNA expression changes on islet cell survival. Dissociated rat (**a**, **c**, **e**) and human (**b**, **d**, **f**) islet cells were transfected with the indicated miRNA mimics (**a**, **b**) or anti-miRNAs (**c**–**f**). Cell death was assessed by scoring the cells displaying pycnotic nuclei on Hoechst staining. Incubation for 24 h with a mix of proinflammatory cytokines was used as a positive control for apoptosis (grey bars). The results correspond to the mean ± SD of three to four independent experiments. *Significantly different from control condition (ctrl or anti-ctrl, as shown), $p \leq 0.05$ by ANOVA analysis, Dunnett's post-hoc test. Ctrl, control; cyt. mix, proinflammatory cytokine mixture

bioinformatics-prediction algorithms (http://mirsystem.cgm.ntu.edu.tw/) and a literature search, we identified other miRNA targets potentially explaining the functional effects observed. In hepatocytes, miR-199a-3p regulates the expression of mTOR and of the transcription factor cMET [21], two proteins known to play important roles in the control of beta cell mass and survival [22, 23]. We found that upregulation of miR-199a-3p results in decreased expression of mTOR and cMET also in MIN6B1 cells (ESM Fig. 6), possibly explaining the negative impact of this miRNA on beta cell survival.

Increased expression of miR-132 displays beneficial effects on both beta cell mass and function. Computational prediction algorithms (http://mirsystem.cgm.ntu.edu.tw/) indicate that granuphilin (also known as synaptotagmin-like 4 [SLP-4]), a granule-associated protein that negatively affects insulin release [16], and GSK-3β, which negatively regulates

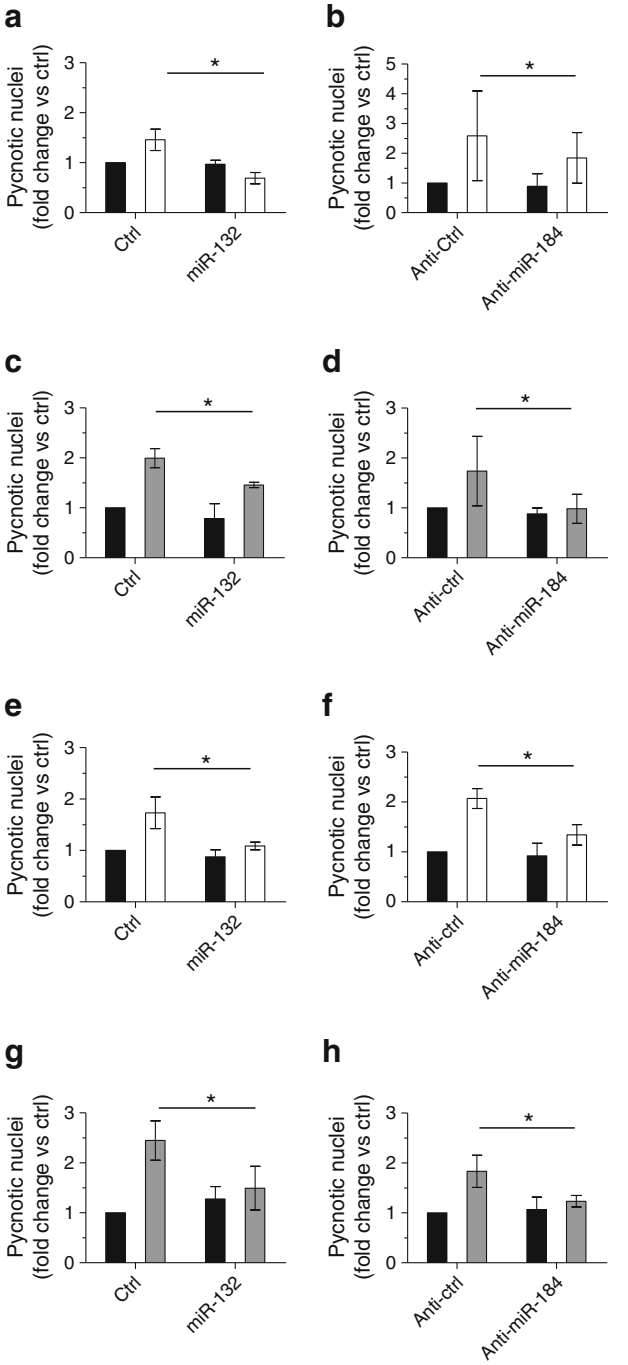

Fig. 7 Overexpression of miR-132 and inhibition of miR-184 protects beta cells against palmitate- or cytokine-induced apoptosis. Dissociated rat (**a**–**d**) or human (**e**–**h**) islet cells were transfected with miR-132 mimic or with anti-miR-184. The cells were then incubated for 48 h with (white bars) or without (black bars) 0.5 mmol/l palmitate coupled to 0.5% BSA (**a**, **b**, **e**, **f**), or for 24 h with (grey bars) or without (black bars) a mix of proinflammatory cytokines (**c**, **d**, **g**, **h**). Apoptosis was assessed at 48 h post-transfection by Hoechst staining of pycnotic nuclei. The results correspond to the mean ± SD of three to four independent experiments. *Significantly different from treated control condition (control or anti-control, as shown), $p \leq 0.05$ by ANOVA analysis, Dunnett's post-hoc test. Ctrl, control

beta cell function and mass [24, 25], are potential miR-132 targets. Translational repression of these two genes could explain, at least in part, the phenotypic traits of beta cells overexpressing miR-132. However, western blot analysis did not reveal any significant impact of miR-132 on the level of these proteins in MIN6B1 cells (ESM Fig. 6). MiRNAs often have small impacts on the expression of single direct targets [26]. However, cumulative effects can have major indirect influences on gene expression and cellular activities. Thus, instead of searching for direct targets, we measured the cellular level of a group of transcription factors known from the literature to improve survival and function of beta cells [27–29]. We found that upregulation of miR-132 in rat islet cells did not affect the mRNA levels of *Foxm1* and *Pdx1* but increased the level of *Mafa* (Fig. 8b). Downregulation of miR-184 that induces overlapping phenotypic changes did not alter the expression level of these genes (not shown).

Discussion

We have identified two groups of miRNAs displaying differential expression in pancreatic islets isolated from two animal models characterised by obesity, insulin resistance and beta cell dysfunction: *db*/*db* mice and HFD-fed mice. The changes in expression of miR-21, miR-34a, miR-132, miR-146a, miR-184, miR-210 and miR-383 detected in this study are consistent with those described by Zhao et al in the islets of leptin-deficient *ob*/*ob* mice [5] and are in agreement with previous findings from our laboratory [6, 8]. Elevated miR-21 levels were also detected in islets of glucose-intolerant human donors [30]. Moreover, our microarray data confirm the upregulation of miR-802 in the islets of *db*/*db* mice recently observed by Kornfeld et al [19]. Increased expression of miR-132, miR-199a-5p and miR-199a-3p have also been reported in the islets of GK rats, a lean model

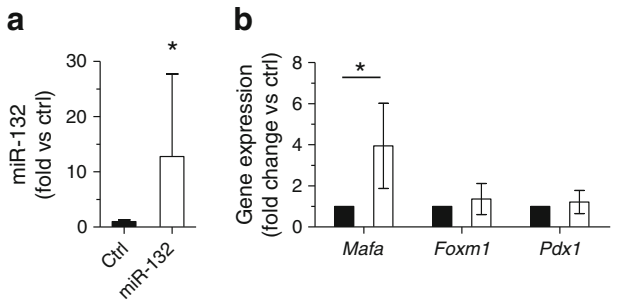

Fig. 8 Effect of mir-132 overexpression on the level of key beta cell transcription factors. Dissociated rat islet cells were transfected with control (black bars) or miR-132 oligonucleotide mimics (white bars). Two days after transfection, the levels of miR-132 (**a**) and *Mafa*, *Foxm1* and *Pdx1* mRNAs (**b**) were analysed by qRT-PCR. The results correspond to the mean ± SD of at least three independent experiments. *Significantly different from control condition, $p \leq 0.05$ by ANOVA analysis, Dunnett's post-hoc test. Ctrl, control

of type 2 diabetes [7]. Consistent with results obtained in *ob*/*ob* mice [5], our microarray data did not reveal significant changes in the level of many miRNAs that play important roles in the control of beta cell functions, including miR-9, miR-24, miR-124a and miR-148 [18, 31–33]. Moreover, we did not detect differences in the levels of miR-375, an islet-enriched miRNA that regulates insulin secretion and beta cell proliferation and that is slightly upregulated (about 30%) in *ob*/*ob* mice [34]. Thus, although appropriate expression of these miRNAs is required for ensuring optimal beta cell function, the development of type 2 diabetes appears not to be associated with major changes in the level of these non-coding RNAs. However, individuals expressing inappropriate levels of these miRNAs may display defective beta cell functions [30] and may be more susceptible to type 2 diabetes manifestation. Indeed, *ob*/*ob* mice lacking miR-375 develop diabetes [34].

The analysis of the functional impact of individual changes in miRNA expression in isolated islet cells revealed that some of them have beneficial effects on the activity of insulin-secreting cells whereas others result in beta cell death. Upregulation of miR-132 and downregulation of miR-184 and miR-338-3p are already observed in 6 week-old prediabetic obese *db*/*db* mice. These adaptive changes in miRNA expression that have a positive impact on beta cell functions are conserved or even more pronounced in HFD-fed and 14–20-week-old diabetic *db*/*db* mice. Indeed, when the level of these particular miRNAs was modulated in vitro, both tumoral and normal beta cells displayed enhanced proliferation and resistance to pro-apoptotic stimuli (present study and Jacovetti et al [9]). Moreover, a rise in the level of miR-132 improved the secretory response of the cells to glucose. These observations suggest that adaptive expression of miR-132, miR-184 and miR-338-3p may contribute to beta cell compensation processes.

The increased miR-132 content and the decreased miR-184 expression observed in *db*/*db* and HFD-fed mice were mimicked by incubation of dissociated rat islet cells in the presence of chronically elevated concentrations of palmitate and glucose. This suggests that these miRNAs may be induced in response to hyperglycaemia and hyperlipidaemia, two conditions typically encountered in prediabetic and diabetic states. In neurons, the expression of miR-132 is triggered following activation of the cAMP-dependent pathway and of the transcription factor cAMP response element-binding protein (CREB) [35–40]. Incubation of rat insulinoma INS-1 832/13 cells with cAMP-raising agents has been shown to cause a rapid increase in the miR-132 precursor [41], indicating that a similar regulatory mechanism may also operate in beta cells.

The mechanisms underlying the effects caused by changes in the level of miR-132 and miR-184 remain to be fully elucidated. We found that upregulation of miR-132 in dissociated rat islet cells leads to increased expression of *Mafa*, a gene playing an important role in the control of beta cell

function and survival [28]. The expression of this transcription factor is decreased by palmitate [42] and is strongly reduced in the islets of diabetic *db*/*db* mice [10, 43]. Moreover, nuclear MAFA was recently reported to be diminished in the islets of individuals affected by type 2 diabetes [44]. Our data suggest that the induction of miR-132 helps preserve the level of MAFA during obesity-associated beta cell compensation.

Over the long term, the adaptive changes elicited by miR-132, miR-184 and miR-338-3p may become insufficient to counterbalance insulin resistance; alterations in the levels of additional miRNAs with deleterious impacts on beta cells also add to the effect. Indeed, the islets of HFD-fed and of diabetic *db*/*db* mice displayed changes in the levels of several other miRNAs, including miR-21, miR-34a, miR-146a, miR-199a-5p, miR-199a-3p, miR-203, miR-210 and miR-383; variation in expression of these miRNAs in vitro causes beta cell dysfunction and death (Lovis et al [6], Roggli et al [8] and present study). We previously showed that induction of miR-34a and miR-146a triggers beta cell apoptosis and that miR-21 and miR-34a have a deleterious impact on insulin secretion [6, 8]. Experiments carried out in this study revealed an increase in apoptosis after overexpression of miR-199a-3p or downregulation of miR-203, miR-210 and miR-383 in dissociated rat and human islet cells and in MIN6B1 cells. These phenotypic changes are not unique to beta cells as modifications in the level of some of these miRNAs promote apoptosis in other cell systems [21, 45–47]. Overexpression of miR-199a-3p resulted in a reduction of the levels of mTOR and cMET, two well-characterised targets of this miRNA [21, 48]. Disruption of the signalling pathways involving these two proteins is detrimental for beta cells [23, 49]. Moreover, mTOR is an important regulator of autophagy, a process thought to contribute to type 2 diabetes onset [50]. Thus, the toxic effects of miR-199a-3p may be the consequence of diminished expression of mTOR and cMET.

In conclusion, the present study is the first globally addressing the role of miRNAs in the aetiology of type 2 diabetes by systematically investigating the impact on primary beta cell function of miRNA changes observed in two animal models of obesity-associated diabetes. Our data demonstrate that obesity and insulin resistance are associated with modifications in two distinct groups of islet miRNAs that have opposing phenotypic effects on beta cells. Expression changes in miRNAs promoting beta cell mass expansion and boosting glucose-induced insulin secretion already occur in normoglycaemic animals and probably belong to adaptive processes allowing beta cells to compensate for insulin resistance. If these mechanisms fail to compensate for the diminished insulin sensitivity, additional modifications in miRNA expression may accumulate, causing beta cell failure and manifestation of type 2 diabetes. We propose that beta cell activities are tuned by a balance between the levels of particular miRNAs associated with enhanced function and mass, such as miR-132, miR-184 and miR-338-3p, and others having negative impacts, including miR-21, miR-34a, miR-146a, miR-199a-5p, miR-199a-3p, miR-203, miR-210 and miR-383. A better understanding of the precise role of particular miRNAs involved in the natural history of the beta cell in diabetes may be harnessed to design novel therapeutic strategies for diabetes prevention and treatment.

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