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Authors: Nesca V, Guay C, Jacovetti C, Menoud V, Peyot ML, Laybutt DR, Prentki M, Regazzi R

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

Valeria Nesca[1]*, Claudiane Guay[1]*, Cécile Jacovetti[1], Véronique Menoud[1], Marie-Line Peyot[2], D. Ross Laybutt[3], Marc Prentki[2] and Romano Regazzi[1]

[1]Department of Fundamental Neurosciences, Faculty of Biology and Medicine, University of Lausanne, Lausanne, Switzerland

[2]Molecular Nutrition Unit and the Montreal Diabetes Research Center at the CRCHUM and the Departments of Nutrition and Biochemistry, University of Montreal, Montreal, QC, Canada

[3]Diabetes and Obesity Research Program, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, New South Wales, Australia

* These authors contributed equally to this work

Correspondence to:

Dr. Romano Regazzi, Department of Fundamental Neurosciences

Rue du Bugnon 9, CH-1005 Lausanne, Switzerland

Tel. : +41 21 692 52 80 / Fax : +41 21 692 52 55 E-mail : Romano.Regazzi@unil.ch

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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 obesity-associated type 2 diabetes models and to assess their potential contribution to the development of the disease.

Methods: MicroRNA profiling of pancreatic islets isolated from pre-diabetic 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 in 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 level 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 level of particular microRNAs to allow sustained beta cell function, and that additional microRNA deregulation negatively impacting on insulin-secreting cells may be causative of 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, Pancreatic islet, microRNA, Obesity, Secretion

Abbreviations:

FoxM1	forkhead box M1
GSK-3β	glycogen synthase kinase 3 beta
HFD	high fat diet
MAFA	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein A
miRNA	microRNA
mTOR	mammalian target of rapamycin
Pdx1	pancreatic and duodenal homeobox 1
PRL	prolactin

INTRODUCTION

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

fatty acids and proinflammatory cytokines [4]. Moreover, alterations in the level 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 analyzed the global variations in islet miRNA expression in pre-diabetic and diabetic *db/db* mice [10] and in high fat diet (HFD) fed mice [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 upon chronic exposure to pro-apoptotic conditions. The results indicate that specific changes in islet miRNA expression in pre-diabetic 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 hyperglycemic states and manifestation of diabetes.

METHODS

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

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 (Australia) [10]. Five-week-old male C57BL/6 mice were purchased from Charles River Laboratories (St-Constant, QC, Canada) and fed normal or high fat diet (Bio-Ser Diet #F3282, Frenchtown, NJ, 60% (wt/wt) fat by energy) during 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 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) from islets of C57BL/KsJ *db/db* mice or of control animals. Total RNA from islets of C57BL/6 mice fed a normal diet or a high fat diet 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 the Agilent Technologies miRNA Gene Microarrays. Microarrays included probes for mouse miRNAs listed on http://www.mirbase.org/ (Release 14, 2009).

Isolation and culture of dissociated islet cells. Pancreatic islets were isolated as described [12] by collagenase digestion followed by purification on a Histopaque (Sigma-Aldrich, St. Louis, MO) density gradient. The islets were first cultured overnight in RPMI 1640 Glutamax medium (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) fetal calf serum (FCS, Amimed, BioConcept AG, Allschwill, Switzerland), 50 U/ml penicillin, 50 µg/ml streptomycin, 1 mmol/l NaPyruvate and 250 µmol/l HEPES and then dissociated by incubation with trypsin (5mg/ml at 37°C during 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 program sponsored by the Juvenile Diabetes Research Foundation. The use of human islets was approved by the Geneva institutional ethical committee. Dissociated human islet cells prepared using the procedure described above were cultured in CMRL medium (Invitrogen, Carlsbad, CA) 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 ESM Table 1.

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

Transfection and modulation of miRNA levels. MIN6B1 and dissociated rat or human islet cells were transfected with Lipofectamine 2000™ (Invitrogen, Carlsbad, CA) 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 siRNA duplex directed against green fluorescent protein (sense 5'-GACGUAAACGGCCACAAGUUC-3' and antisense 5'-ACUUGUGGCCGUUUACGUCGC-3') and the miScript miRNA reference inhibitor (Qiagen, Hombrechtikon, Switzerland) were used as negative controls for miRNA overexpression and down-regulation, respectively.

Measurement of miRNA and mRNA expression. Mature miRNA expression was assessed with the miRCURY LNA™ Universal RT microRNA PCR kit (Exiqon, Vedbaek, Denmark). Measurements of the level of putative target mRNAs were performed by conventional reverse transcription (Promega, Dübendorf, Switzerland) followed by quantitative PCR (Biorad,

Reinach, Switzerland) with custom-designed primers (Microsynth, Balgach, Switzerland) that are available upon request. MiRNA expression was normalized to the level of U6 or miR-7 (an islet-specific miRNA used as internal control) while mRNA expression was normalized to 18S.

Insulin secretion. Two days after transfection, MIN6B1 or dissociated rat islet cells were pre-incubated during 30 min at 37°C in KREBS buffer (127 mmol/l NaCl, 4.7 mmol/l KCl, 1mmol/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 condition). After gathering the supernatants, the cells were incubated for 45 min in KREBS buffer containing 20 mmol/l glucose (stimulatory condition). The incubation medium was collected and total cellular insulin contents recovered in EtOH acid (75 % (vol/vol) EtOH, 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, Carlsbad, CA) during 1 min. The fraction of cells (at least 1 x 10^3 per condition) displaying pycnotic nuclei was scored under fluorescence microscopy (AxioCam MRc5, Zeiss). Apoptosis was triggered by exposing the cells during 24h to cytokines (30 ng/ml INFγ, 10 ng/ml TNFα and 0.1 ng/ml IL-1β) or during 48h to medium (5 % (vol/vol) FCS) supplemented with 0.5 mmol/l palmitate bound to 0.5 % (wt/vol) BSA [15].

Proliferation assay. Transfected MIN6B1 or dissociated islet cells cultured on poly-L-lysine coated glass coverslips were fixed with ice-cold methanol and permeabilized with 0.5% (wt/vol) saponin (Sigma-Aldrich, St. Louis, MO). The coverslips 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, Carlsbad, CA). At the end of the incubation, the coverslips were washed with PBS containing Hoechst 33342 (Invitrogen, Carlsbad, CA) and images of at least $1x10^3$ cells per condition were collected on a fluorescence microscope. Prolactin (PRL 500 ng/ml during 48h) was used as positive control.

Protein extraction and western blotting. Protein lysates (30-50 μg) from MIN6B1 cells prepared as described [9] were separated on polyacrylamide gels and transferred to polyvinylidine fluoride membranes. The membranes were incubated overnight with antibodies against Granuphilin ([16], 1:2000); MAFA (Abcam #ab17976, 1:1000), mTOR (Cell Signaling #2972, 1:1000), cMET (Cell Signaling #3127, 1:1000) and GSK-3β (Cell Signaling #9315, 1:1000). Antibodies against α-Tubulin (T9026, 1:10000, Sigma-Aldrich) and Actin (Clone C4 MAB1501, 1:15000, Millipore) were used to verify equal loading. After one hour exposure to IRDye (Li-Cor® Biosciences, Bad Homburg, Deutschland) or horseradish peroxidase-coupled secondary antibodies, the bands were visualized 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, with one-way analysis of variance (ANOVA) of the means, followed by a post-hoc Dunnett test (SAS statistical package; SAS, Carry, NC).

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 hyperglycemia and beta cell dysfunction after being fed a high fat diet (HFD) for 8 weeks [11]. The characteristics of the animals used in this study are presented in ESM 2-4. We identified more than 60 differentially expressed miRNAs in *db/db* and HFD-fed mice islets compared to their respective controls, with overlapping changes in the two models (Fig.1).

For *db/db* mice, miRNA expression was determined both in pre-diabetic (6 weeks old) and diabetic (14-20 weeks old) animals. In pre-diabetic mice, the miRNAs displaying the most striking changes were miR-132, whose expression increased by 8.2-fold, and miR-210, miR-184, and miR-203 that decreased by 4.0, 3.4 and 2.0 fold, respectively (ESM Table 5). In agreement with our previous findings [9], the islets of pre-diabetic *db/db* mice contained also 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 up-regulation of miR-132 and down-regulation of miR-203 and miR-338-3p remained approximately constant in pre-diabetic and diabetic animals (ESM Table 5 and 6). In addition to these changes, the islets of adult diabetic mice were characterized by alterations in the level of additional miRNAs, including an up-regulation of miR-199a-5p (12.6 fold) and miR-199a-3p (9.3 fold), a decline of miR-383 (13.7 fold) and, as previously reported [6], an increase of miR-34a and miR-146a (ESM Table 6). The results obtained by microarray analysis were confirmed by quantitative RT-PCR (Fig.2a-l). Our microarray data revealed also an increase of miR-21 (2.2 fold), that we have previously shown to inhibit insulin secretion [8], a decrease of miR-26a (1.6 fold) that controls insulin biosynthesis [18] and an increase of miR-802 regulating *Hnf1b* expression [19] (ESM Table 5 and 6). The role of these miRNAs was not further investigated in this study.

Islet miRNA expression was also analyzed in HFD-fed mice. For this purpose we selected a group of mice displaying the strongest response to HFD. These animals were markedly obese, insulin resistant, hyperinsulinemic and clearly hyperglycemic (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 (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 hyperglycemia.

miRNA expressions are 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 level of these non-coding RNAs is affected by chronic exposure of beta cells to elevated concentration of glucose and free fatty acids. We found that prolonged incubation of rat islets (Fig.3) under glucolipotoxic conditions mimicked the modifications in miR-132, miR-184, miR-199-3p, 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

MicroRNA expression modifications in islets could reflect the activation of adaptive processes counterbalancing the increased insulin needs caused by obesity and insulin resistance or the instauration of pathological conditions leading to beta cell dysfunction. Indeed, we have previously shown that down-regulation of miR-338-3p contributes to compensatory beta cell mass expansion [9], whereas overexpression of miR-21, miR-34a and miR-146a negatively impacts beta cell function [6]. 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-miR molecules that specifically inhibit miRNAs (ESM Fig.1). The transfected cells were then analyzed 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, up-regulation 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, up-regulation of miR-132 or down-regulation of miR-184, miR-203 and miR-383 led to an increase in proliferation while modifications of the level of other miRNAs had no significant effects (ESM Fig.3). Proliferation of insulin-positive cells was also observed upon up-regulation of miR-132 and, to a lesser extent, down-regulation of miR-184 in dispersed rat islet cells (Fig.5). In contrast, down-regulation of miR-203 and miR-383 in primary cells had no effect. Similar to our previous work with miR-338-3p [9], these findings suggest that modifications of the level of miR-132 and miR-184 contributes to compensatory beta cell mass expansion elicited in response to insulin resistance.

Since 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-21, miR-34a and miR-146a

[6], up-regulation 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 free fatty acids 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 characterized 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 Bcl2 [6]. Combining bioinformatics prediction algorithms (http://mirsystem.cgm.ntu.edu.tw/), and literature search, we identified other miRNA targets potentially explaining the functional effects observed. In hepatocytes, miR-199a-3p regulates the expression of mammalian target of rapamycin (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 up-regulation 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 Slp-4), a granule-associated protein that negatively affects

insulin release [16], and GSK-3β, which negatively regulates 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 up-regulation of miR-132 in rat islet cells did not affect the mRNA levels of *FoxM1* and *Pdx-1* but increased the level of *MafA* (Fig.8b). miR-184 down-regulation 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 characterized by obesity, insulin resistance and beta cell dysfunction: the *db/db* mice and the HFD-fed mice. The changes in the 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 and coll. in the islets of leptin-deficient *ob/ob* mice [5] and are in agreement with previous findings from our laboratory [6, 9]. Elevated miR-21 levels were also detected in islets of glucose-intolerant human donors [30]. Moreover, our microarray data confirm the up-regulation 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 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-124 and miR-148 [18, 31-33]. Moreover, we did not detect differences in the level of miR-375, an islet enriched miRNA that regulates insulin secretion and beta cell proliferation and that is slightly up-regulated (about 30%) in *ob/ob* mice [34]. Thus, although appropriate expression of these miRNAs is required for insuring optimal beta cell functions, 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. Up-regulation of miR-132 and down-

regulation of miR-184 and miR-338-3p are already observed in 6 week-old pre-diabetic obese *db/db* mice. These adaptive changes in miRNA expression that have a positive impact on beta cell functions are conserved or are even more pronounced in HFD-fed and 14 to 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 [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 hyperglycemia and hyperlipidemia, two conditions typically encountered in pre-diabetic and diabetic states. In neurons, the expression of miR-132 is triggered following activation of the cAMP-dependent pathway and of the transcription factor CREB [35-40]. Incubation of INS-1 832/13 cells, a rat insulinoma cell line, with cAMP-raising agents has been shown to cause a rapid increase of 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 up-regulation 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 preserving the level of MafA during obesity associated beta cell compensation.

On the long term, the adaptive changes elicited by miR-132, miR-184 and miR-338-3p may become insufficient to counterbalance insulin resistance, also because of alterations in the level of additional miRNAs having deleterious impacts on beta cells. Indeed, the islets of HFD-fed and of diabetic *db/db* mice displayed changes in the level of several other miRNAs, including miR-21, miR-34a, miR-146a, miR-199a-5p, miR-199a-3p, miR-203, miR-210 and miR-383 whose variation in expression *in vitro* causes beta cell dysfunction and death ([6] [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]. Experiments carried out in this study revealed an increase in apoptosis upon overexpression of miR-199a-3p or down-regulation 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 since 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 characterized targets of this miRNA [21, 48]. Disruption of the signaling 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 etiology 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 normoglycemic 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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FIGURE LEGENDS

FIG 1. miRNAs differentially expressed in pancreatic islets of type 2 diabetes animal models. Pancreatic islets were isolated from prediabetic (6 weeks old) and diabetic (14-20 weeks old) *db/db* mice and from high-fat diet fed (HFD) mice. miRNA expression levels were analysed by microarray. Data are expressed as fold changes *versus* the corresponding controls and are presented on a logarithmic scale. Only miRNAs displaying increases (white circles) or decreases (black squares) of at least 1.5 fold ($p \leq 0.05$) are plotted. Dashed lines correspond to 2 fold change.

FIG 2. miRNA expression changes in islets of type 2 diabetes animal models. The expression level of the indicated miRNAs was measured by qRT-PCR in pancreatic islets of prediabetic (**a-d**) and diabetic (**e-l**) *db/db* mice (*versus* age-matched mice) and in mice fed a normal or a high-fat diet (**m-s**). The results correspond to the mean ± SD of three to four animals per group and are normalized to the level of the respective controls. * Significantly different from control ($p \leq 0.05$ by unpaired student t-test).

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 (11 G) or 20 (20 G) mmol/l glucose with 0.5% BSA in the absence (black bars) or presence of 0.5 mmol/l palmitate for 48h (grey bars) or 72h (white bars). miRNA expression levels were measured by qRT-PCR, normalized by miR-7 and expressed as percentage of control (11 mmol/l glucose with 0.5 % BSA) (**a-g**). * Significantly different from the control condition ($p \leq 0.05$ by ANOVA analysis, Dunnett's post-hoc test).

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 down-regulation (**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 48h post-transfection. Insulin release is expressed as percentage of insulin content. * Significantly different from control condition (*versus* control transfection, incubated at the same glucose concentration) ($p \leq 0.05$ by ANOVA analysis, Dunnett's post-hoc test).

FIG 5. Effect of specific modifications in miRNA expression on β-cell proliferation. Dispersed rat islet cells were transfected with oligonucleotides leading to overexperession (**a**) or down-regulation (**b**) of the indicated miRNAs. β−cell proliferation was assessed 72h later by the staining of the cells with anti-Ki67 and anti-insulin antibodies. Prolactin (PRL 500 ng/ml during 48h, cross-hatched bar) was used as positive control. The results correspond to the mean ± SD of three to six independent experiments. * Significantly different from control condition ($p \leq 0.05$ by ANOVA analysis, Dunnett's post-hoc test).

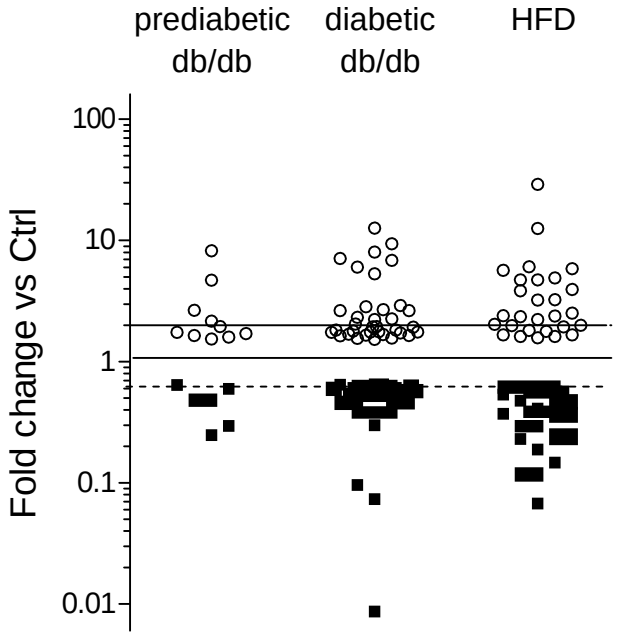
FIG 6. Impact of specific miRNA expression changes on islet cell survival. Dissociated rat (**a**, **c**, **e**) and human (**b**, **d**, **f**) islet cells were transfected with the indicated miRNA mimics (**a**, **b**) or anti-miRNAs (**c**, **d**, **e**, **f**). Cell death was assessed by scoring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

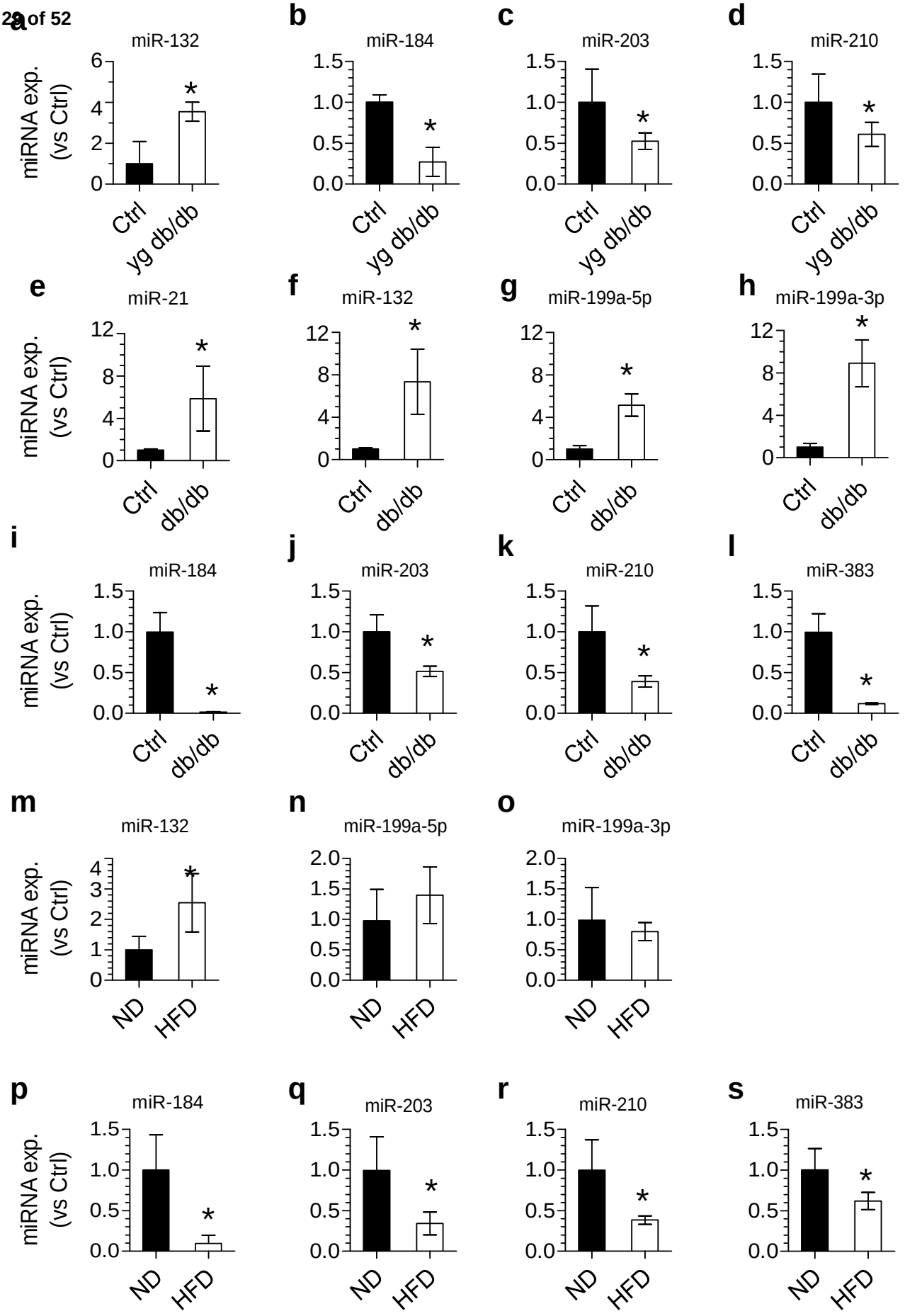
FIG 7. Overexpression of miR-132 and inhibition of miR-184 protect β-cells against palmitate or cytokines-induced apoptosis. Dissociated rat (**a-d**) or human (**e-h**) islet cells were transfected with miR-132 mimic, or with anti-miR-184. The cells were then incubated for 48h with (white bars) or without (black bars) 0.5 mmol/l palmitate coupled to 0.5 % BSA (**a**, **b**, **e**, **f**), or for 24h with (cross-hatched bars) or without (black bars) a mix of pro-inflammatory cytokines (**c**, **d**, **g**, **h**). Apoptosis was assessed 48h 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. ($p \leq 0.05$ by ANOVA analysis, Dunnett's post-hoc test).

FIG 8. Effect of mir-132 overexpression on MafA mRNA expression level in islet cells. Dissociated rat islet cells were transfected with control (black bars) or miR-132 oligonucleotide mimics (white bars). Two days after transfection the level of miR-132 (**a**) and of 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).

Nesca et al. Figure 1




a
miR-132
miRNA exp. (vs Ctrl)
Ctrl
yg db/db
b
miR-184
c
miR-203
d
miR-210
e
miR-21
db/db
f
miR-132
g
miR-199a-5p
h
miR-199a-3p
i
miR-184
j
miR-203
k
miR-210
l
miR-383
m
miR-132
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miR-184
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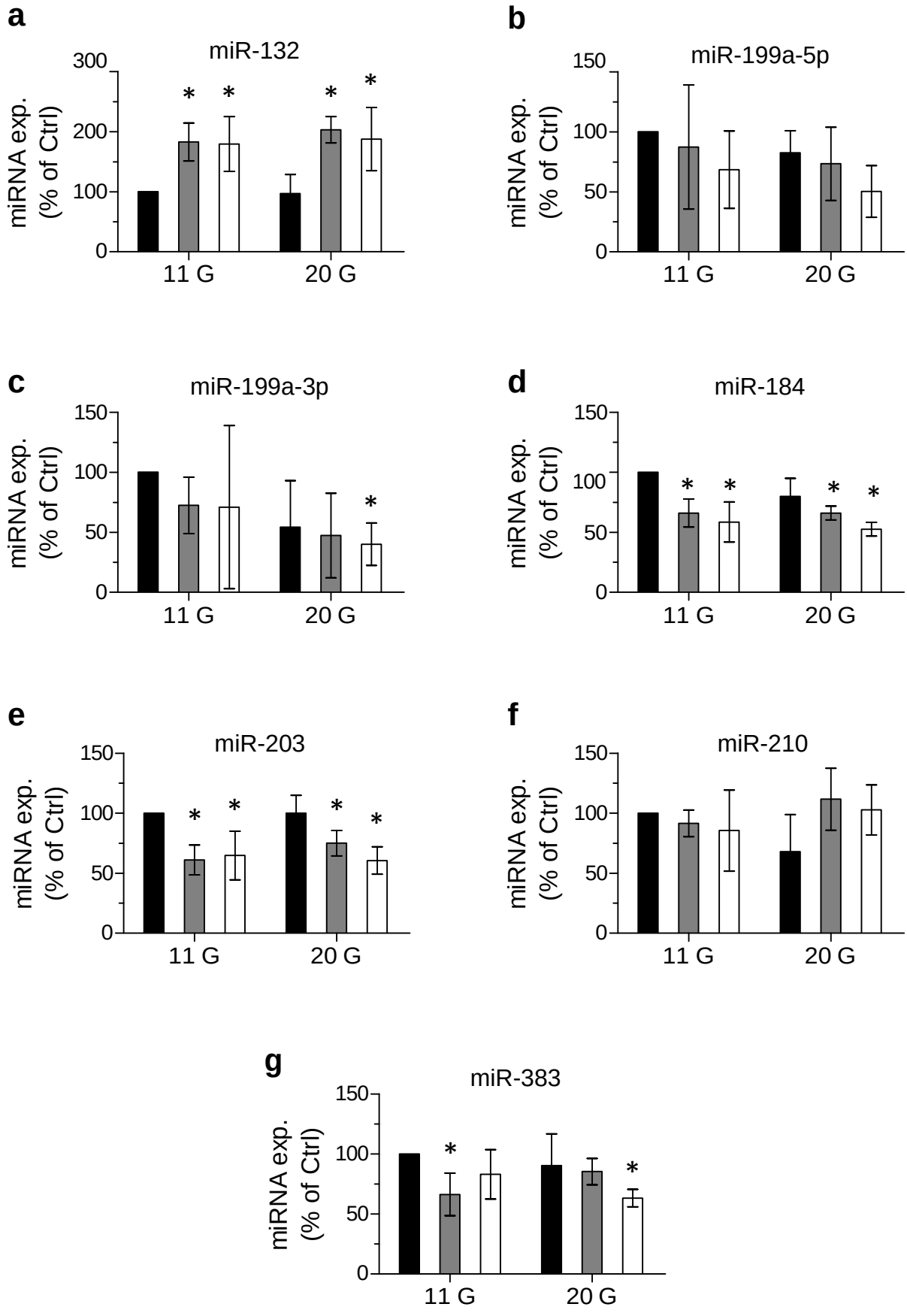
miR-210
s
miR-383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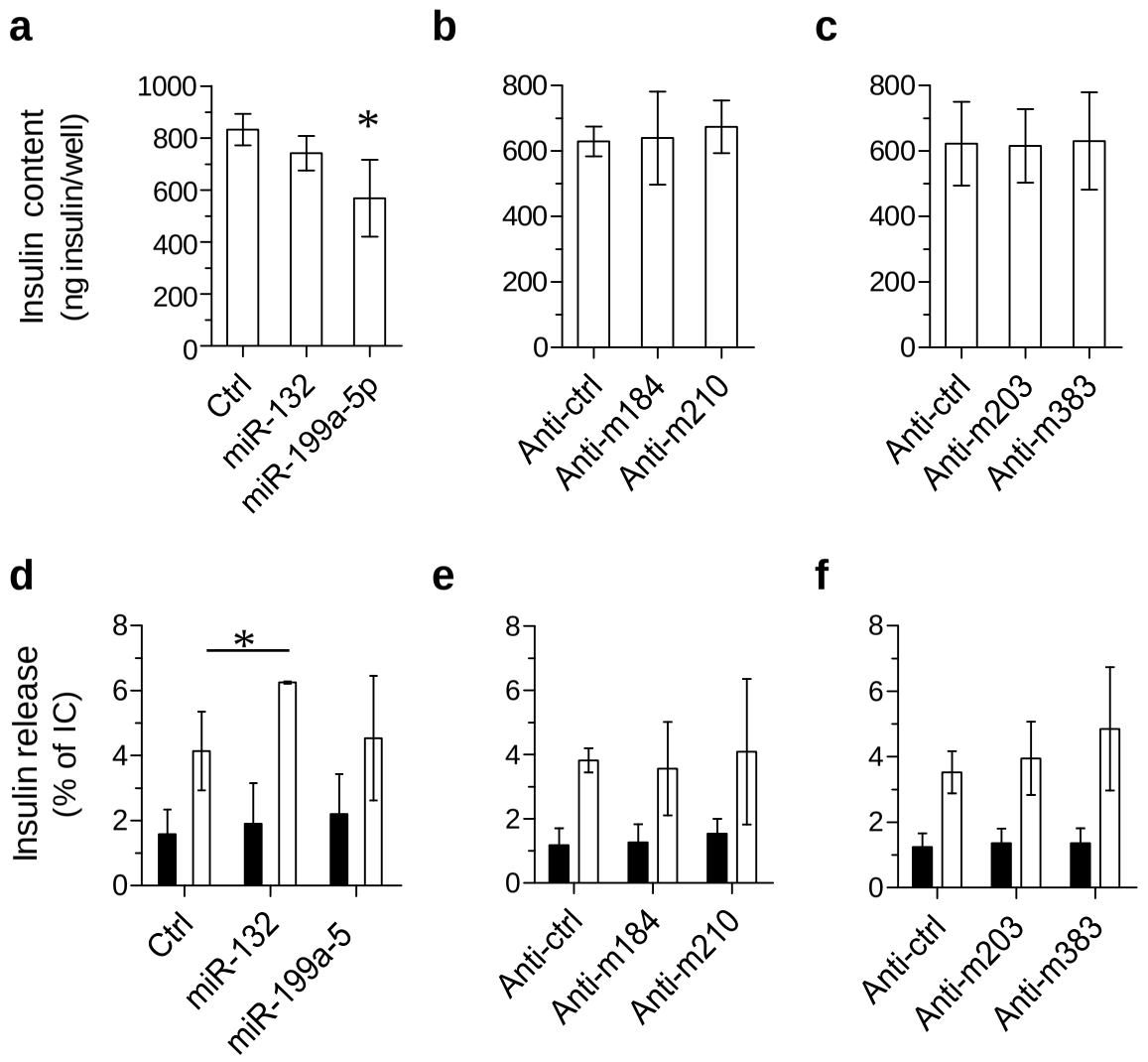
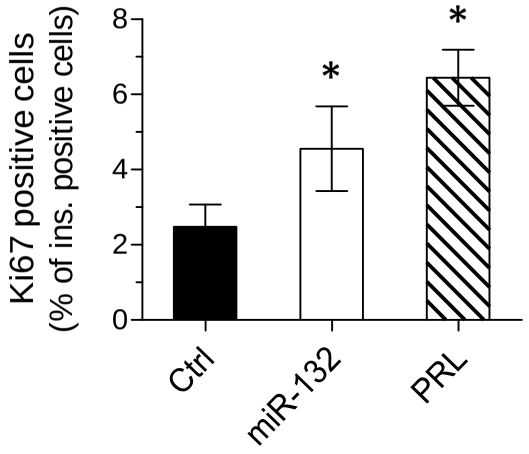
miR-132
miRNA exp. (% of Ctrl)
300
200
100
0
11 G
20 G
b
miR-199a-5p
150
100
50
c
miR-199a-3p
d
miR-184
e
miR-203
f
miR-210
g
miR-383

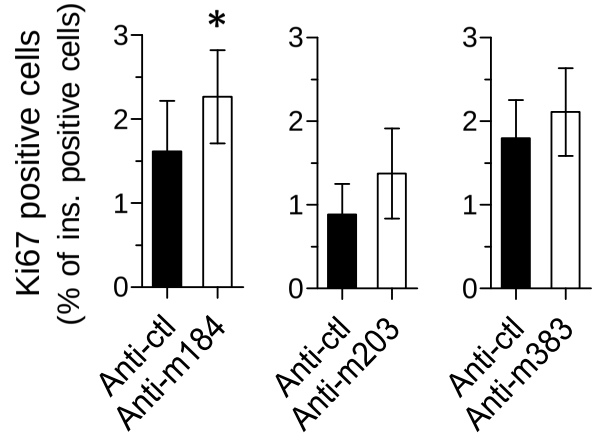
Nesca et al. Figure 4



a


Ki67 positive cells
(% of ins. positive cells)
0
2
4
6
8
*
*
Ctrl
miR-132
PRL

b


Ki67 positive cells
(% of ins. positive cells)
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1
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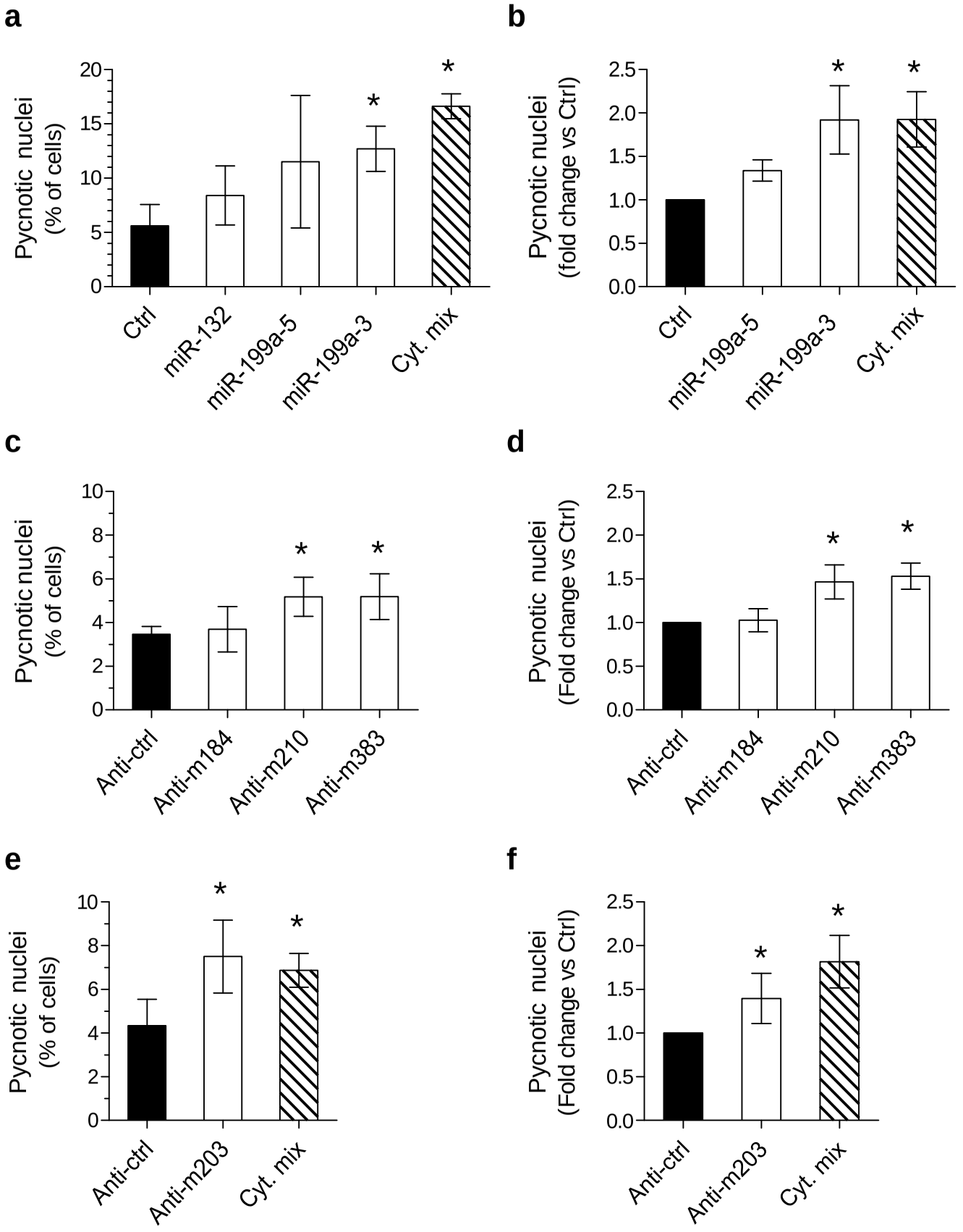
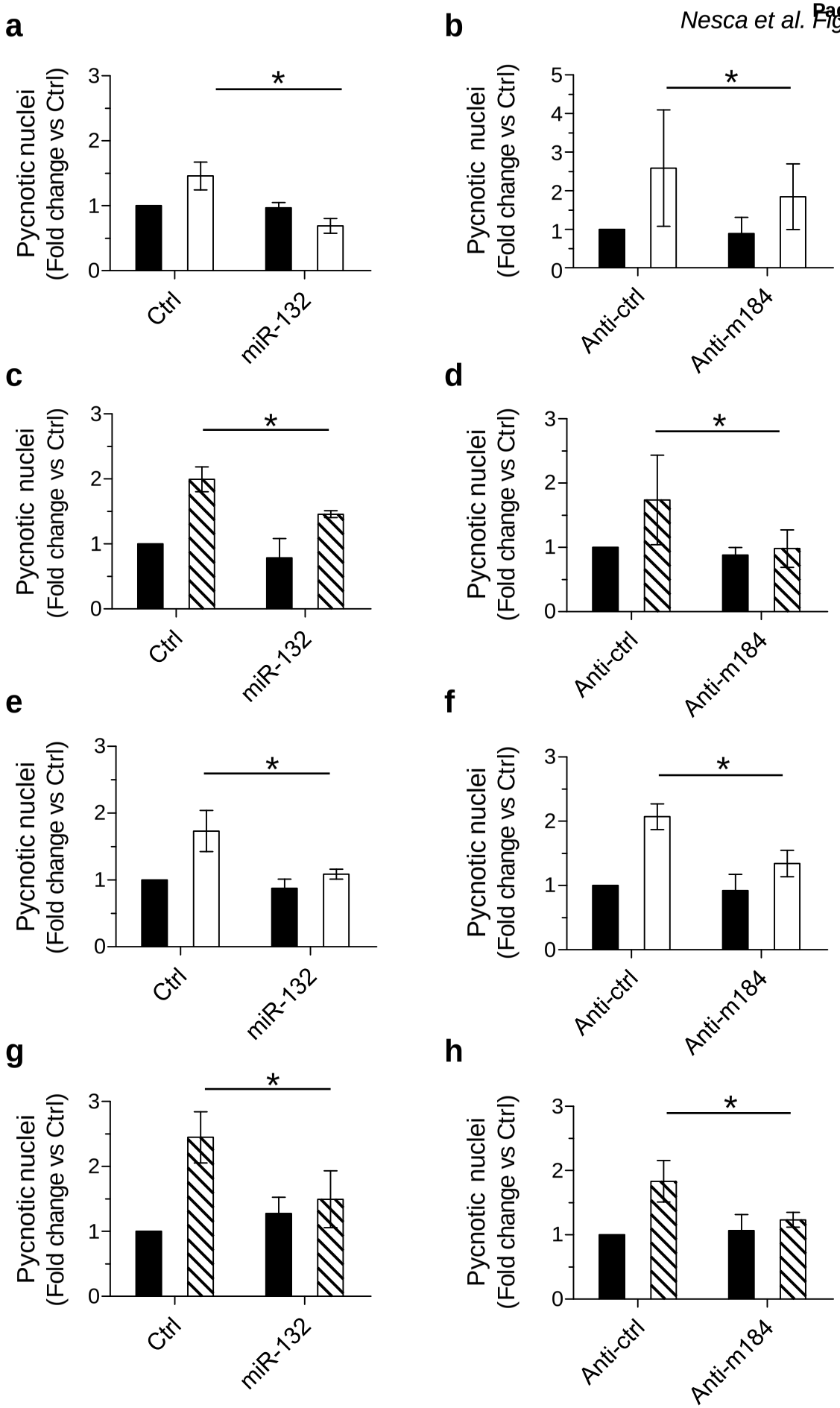
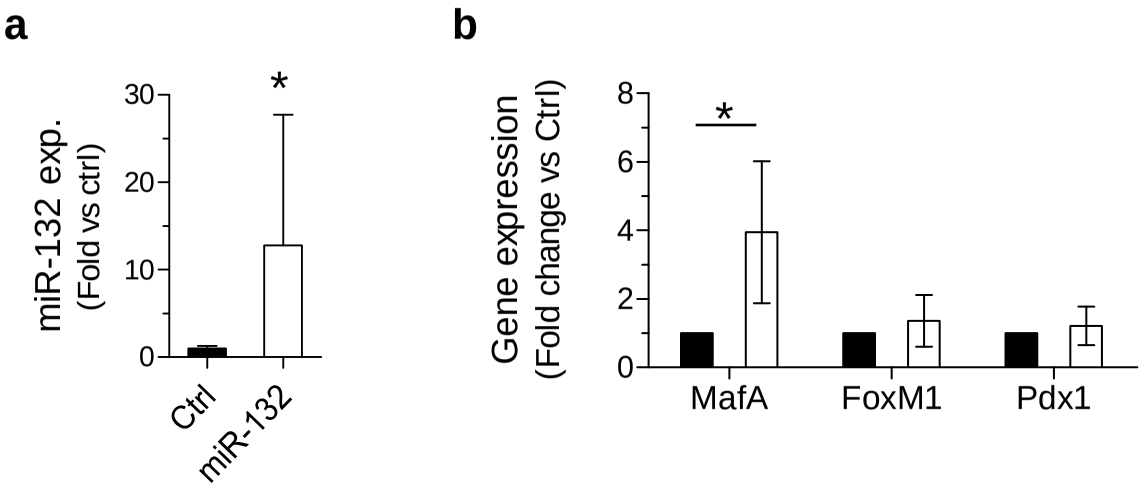
3
*
Anti-ctl
Anti-m184
Anti-ctl
Anti-m203
Anti-ctl
Anti-m383

Nesca et al. Figure 6




a
b
c
d
e
f
g
h
Pycnotic nuclei
(Fold change vs Ctrl)
Ctrl
miR-132
Anti-ctrl
Anti-m184
*

Nesca et al. Figure 8



ESM Table 1: Characteristics of the donors and of the human pancreatic islet preparations that were used for functional assays in this study.

Gender	Age	BMI	Cause of death	Cold ischemic time (hh:mm)	Islets purity (%)	Islets viability (%)
F	54	30.5	n/a	n/a	70	90
F	49	22.0	stroke	01:40	66	85
F	54	19.1	stroke	08:10	79	85
F	n/a	19.1	cerebral trauma	03:37	80	90
F	56	19.7	stroke	07:13	87	90
F	56	23.8	stroke	01:00	85	90
F	58	19.2	stroke	06:00	85	90
F	62	25.7	stroke	03:00	61	95
M	55	25.8	n/a	10:00	89	90
M	49	21	stroke	06:00	93	95
mean	54.8	22.6	-	-	79.5	90.0
SD	4.1	3.8			10.6	3.3

ESM Table 2: characteristics of pre-diabetic *db/db* mice and age-matched controls used in this study. The table shows the weights and glycaemia of each of the mice from which pancreatic islet RNA was isolated. The islet preparations used for microarray analysis are indicated by the asterisks. Insulin plasma levels of an identical population of pre-diabetic *db/db* mice were reported previously [Chan JY et al., Diabetes 62:1557-1568, 2013].

Mice group	Phenotype	Weight (g)	Glycaemia (mmol/l)
Control	db/+	20.7	11.5
	db/+ (*)	20.0	9.7
	db/+	17.3	8.7
	db/+ (*)	15.6	9.1
	db/+ (*)	16.7	7.4
	db/+ (*)	15.3	7.2
mean		17.6	8.9
SD		2.3	1.6
Pre-diabetic db/db	db/db (*)	28.8	8.7
	db/db (*)	30.7	8.5
	db/db	24.2	9.8
	db/db (*)	25.8	11.2
	db/db (*)	25.7	8.8
	db/db	26.3	9.2
	db/db	25.6	9.0
mean		26.7	9.3
SD		2.2	0.9

ESM Table 3: characteristics of diabetic *db/db* mice and age-matched controls used in this study. The table shows the weights and glycaemia of each of the mice from which pancreatic islet RNA was isolated. The islet preparations used for microarray analysis are indicated by the asterisks. Insulin plasma levels of an identical population of diabetic *db/db* mice were reported previously [Chan JY et al., Diabetes 62:1557-1568, 2013].

Mice group	Phenotype	Weight (g)	Glycaemia (mmol/l)
Control	wt (*)	23.0	7.5
	wt	21.1	6.1
	wt	22.5	7.2
	wt	20.8	6.8
	wt (*)	25.5	6.9
	db/+ (*)	26.5	6.8
	db/+ (*)	24.7	6.8
mean		23.4	6.9
SD		2.2	0.4
Diabetic db/db	db/db (*)	50.5	25.5
	db/db	48.1	23.9
	db/db	45.9	17.1
	db/db (*)	47.3	21.3
	db/db	45.1	17.6
	db/db (*)	50.0	20.8
	db/db (*)	47.0	20.2
mean		47.7	20.9
SD		2.0	3.1

ESM Table 4: characteristics of ND and HFD mice used in this study. We selected mice that displayed the highest response to high fat feeding [Peyot ML et al., Diabetes 59: 2178-2187, 2010]. The table reports weights, glycaemia and insulinemias of each of the mice from which pancreatic islet RNA was isolated.

Mice group	Weight before diet (g)	Weight at 6.5 weeks (g)	Glycemia at 6.5 weeks (mmol/l)	Weight at sacrifice (g)	Glycemia at sacrifice (mmol/l)	Insulinemia (mmol/l)
ND	23.10	35.90	7.44	37.70	7.10	437.30
	22.60	33.10	7.55	34.80	6.99	778.59
	22.30	33.10	7.79	34.70	8.49	230.41
	20.90	33.70	7.09	36.00	7.59	414.93
mean	22.23	33.95	7.47	35.80	7.55	465.31
SD	0.94	1.33	0.29	1.40	0.68	228.51
HFD	19.80	46.20	9.05	46.20	9.21	801.34
	22.00	45.80	9.79	47.40	11.69	4916.68
	22.60	48.80	12.19	49.70	11.89	818.46
	23.40	45.30	9.89	47.60	9.09	3324.02
mean	21.95	46.53	10.23	47.73	10.47	2465.12
SD	1.54	1.56	1.36	1.45	1.53	2018.87

ESM Table 5. Microarray analysis of pre-diabetic *db/db vs* control mice. MiRNAs expression was assessed in pancreatic islets of pre-diabetic *db/db* mice *versus* age-matched control animals via microarray analysis. The table shows miRNAs whose expression was significantly different in pre-diabetic *db/db* mice (p-value ≤ 0.05; ≥ 1.5 fold-change difference). The mean expression of each group (control or pre-diabetic *db/db*) is presented in a log2 scale. MiRNAs investigated in this study are highlighted in blue while those studied in previous papers [Lovis et al., Diabetes 57: 2728-2736, 2008; Roggli et al., Diabetes, 59: 978-986, 2010; Jacovetti et al., J Clin Invest, 122: 3541-3551, 2012] are in grey.

Systematic Name	Fold change ([dbdb] vs [ctrl])	Regulation ([db/db] vs [ctrl])	means of expression	
			ctrl	pre-diabetic db/db
mmu-miR-132	8.212	up	6.916	9.954
mmu-miR-455*	4.693	up	2.406	4.637
mmu-miR-152	2.652	up	8.623	10.030
mmu-miR-455	2.407	up	3.614	4.882
mmu-miR-139-5p	2.395	up	1.974	3.234
mmu-miR-301b	2.351	up	1.575	2.808
mmu-miR-721	2.276	up	3.970	5.157
mmu-miR-212	2.158	up	6.747	7.856
mmu-miR-150	2.060	up	2.312	3.355
mmu-miR-483	2.024	up	2.748	3.766
mmu-miR-142-3p	1.943	up	6.277	7.236
mmu-miR-146a	1.941	up	5.442	6.399
mmu-miR-337-5p	1.738	up	7.051	7.848
mmu-miR-193	1.727	up	2.755	3.543
mmu-miR-337-3p	1.715	up	7.816	8.594
mmu-miR-582-5p	1.698	up	4.536	5.300
mmu-miR-146b	1.672	up	3.093	3.835
mmu-miR-182	1.645	up	4.200	4.918
mmu-miR-433	1.594	up	5.253	5.926
mmu-miR-452	1.585	up	4.996	5.661
mmu-miR-676	1.543	up	3.500	4.126
mmu-miR-22	1.541	up	10.727	11.351
mmu-miR-141*	1.523	up	4.573	5.180
mmu-miR-216a	4.200	down	3.268	1.197
mmu-miR-210	4.048	down	4.377	2.360
mmu-miR-762	4.032	down	2.043	0.031
mmu-miR-31*	3.711	down	4.301	2.409
mmu-miR-184	3.385	down	7.165	5.406
mmu-miR-218	3.090	down	4.126	2.499
mmu-miR-23b	2.124	down	11.177	10.090
mmu-miR-203	2.046	down	5.235	4.203
mmu-miR-802	1.794	down	4.709	3.866
mmu-miR-378	1.680	down	4.774	4.025
mmu-miR-671-5p	1.653	down	5.678	4.953
mmu-miR-27b	1.634	down	9.966	9.258
mmu-miR-100	1.619	down	3.925	3.230
mmu-miR-24-1*	1.609	down	3.882	3.195
mmu-miR-338-3p	1.560	down	7.949	7.308
mmu-miR-194	1.503	down	6.222	5.635

ESM Table 6. Microarray analysis of diabetic *db/db vs* control mice. RNA isolated from pancreatic islets of diabetic *db/db* and age-matched control mice was used to profile miRNAs expression by microarray analysis. The table shows miRNAs whose expression was significantly different in diabetic *db/db* mice (p-value ≤ 0.05; ≥ 1.5 fold-change difference). The mean expression of each group (control or pre-diabetic *db/db*) is presented in a log2 scale. MiRNAs investigated in this study are highlighted in orange while those studied previously [Lovis et al., Diabetes 57: 2728-2736, 2008; Roggli et al., Diabetes, 59: 978-986, 2010; Jacovetti et al., J Clin Invest, 122: 3541-3551, 2012] are in grey.

Systematic Name	Fold change ([db/db] vs [ctrl])	Regulation ([db/db] vs [ctrl])	means of expression	
			ctrl	diabetic db/db
mmu-miR-199a-5p	12.615	up	2.534	6.191
mmu-miR-199a-3p	9.359	up	4.745	7.972
mmu-miR-139-5p	8.009	up	1.531	4.532
mmu-miR-199b*	7.095	up	3.824	6.651
mmu-miR-455*	6.841	up	1.958	4.732
mmu-miR-802	6.020	up	4.867	7.457
mmu-miR-132	5.307	up	6.823	9.231
mmu-miR-721	2.902	up	5.122	6.659
mmu-miR-1224	2.830	up	9.254	10.755
mmu-miR-100	2.684	up	3.881	5.305
mmu-miR-497	2.640	up	6.609	8.010
mmu-miR-676	2.634	up	3.558	4.955
mmu-miR-143	2.317	up	5.929	7.141
mmu-miR-21	2.249	up	9.918	11.087
mmu-miR-34b-5p	2.228	up	8.252	9.407
mmu-miR-195	2.042	up	7.148	8.178
mmu-miR-10b	1.953	up	5.423	6.389
mmu-miR-34a	1.932	up	7.372	8.322
mmu-miR-146a	1.924	up	5.969	6.914
mmu-miR-34c	1.819	up	6.805	7.668
mmu-miR-126-3p	1.817	up	10.328	11.189
mmu-miR-322	1.789	up	5.704	6.544
mmu-miR-152	1.766	up	9.210	10.031
mmu-miR-22*	1.751	up	4.488	5.296
mmu-miR-337-5p	1.748	up	7.203	8.009
mmu-miR-582-5p	1.738	up	5.229	6.027
mmu-miR-433*	1.718	up	3.287	4.067
mmu-miR-337-3p	1.678	up	7.886	8.633
mmu-miR-181c	1.674	up	4.933	5.677
mmu-miR-10a	1.648	up	6.182	6.902
mmu-miR-365	1.640	up	5.239	5.953
mmu-miR-146b	1.627	up	3.932	4.634
mmu-miR-320	1.564	up	4.845	5.490
mmu-miR-99a	1.550	up	6.363	6.995
mmu-miR-212	1.535	up	7.010	7.628

mmu-miR-184	115.155	down	6.909	0.061
mmu-miR-383	13.675	down	4.891	1.118
mmu-miR-210	10.427	down	4.709	1.327
mmu-miR-31	3.349	down	7.415	5.671
mmu-miR-203	2.649	down	5.581	4.176
mmu-miR-325	2.418	down	3.984	2.710
mmu-miR-23b	2.262	down	11.329	10.152
mmu-miR-338-3p	2.236	down	8.366	7.206
mmu-miR-378	2.223	down	4.830	3.678
mmu-miR-384-5p	2.139	down	7.084	5.987
mmu-miR-27b	2.055	down	10.095	9.056
mmu-miR-374	1.934	down	5.641	4.689
mmu-miR-328	1.929	down	5.499	4.551
mmu-miR-381	1.840	down	6.586	5.706
mmu-miR-30d	1.804	down	8.426	7.575
mmu-miR-30e*	1.719	down	5.588	4.806
mmu-miR-129-3p	1.711	down	10.830	10.055
mmu-miR-204	1.702	down	8.392	7.625
mmu-miR-434-3p	1.688	down	9.078	8.322
mmu-miR-301a	1.673	down	7.877	7.135
mmu-miR-324-5p	1.648	down	7.110	6.389
mmu-miR-872	1.638	down	6.156	5.444
mmu-miR-30e	1.636	down	9.670	8.960
mmu-miR-103	1.630	down	9.540	8.835
mmu-miR-26a	1.592	down	12.056	11.385
mmu-miR-331-3p	1.586	down	7.048	6.383
mmu-miR-652	1.585	down	8.935	8.270
mmu-miR-341	1.570	down	6.075	5.424
mmu-miR-129-5p	1.548	down	7.979	7.349
mmu-miR-324-3p	1.538	down	5.085	4.464

ESM Table 7. Microarray analysis of miRNA expression in pancreatic islets from mice fed a normal or a high fat diet. MiRNA expression was profiled in pancreatic islets isolated from mice fed a high fat diet (HFD) or normal diet (ND) during 8 weeks. The table shows all the miRNAs displaying expression changes under high fat diet higher than 1.5 fold. The miRNAs investigated in this study are highlighted in green.

Systematic Name	Fold change ([HFD] vs [ND])	Regulation ([HFD] vs [ND])	means of expression	
			ND	HFD
mmu-miR-125a-3p	28.959	up	4.689	9.545
mmu-miR-211	12.573	up	3.336	6.989
mmu-miR-714	12.573	up	5.120	8.772
mmu-miR-221	6.039	up	5.453	8.048
mmu-miR-7a-1*	5.830	up	12.186	14.730
mmu-miR-380-3p	5.671	up	3.393	5.897
mmu-miR-802	5.044	up	3.336	5.671
mmu-miR-200c*	4.893	up	5.634	7.924
mmu-miR-1897-5p	4.727	up	3.460	5.701
mmu-miR-188-5p	4.720	up	3.594	5.833
mmu-miR-193	4.031	up	3.336	5.347
mmu-miR-132	3.923	up	8.053	10.025
mmu-let-7d*	3.835	up	7.507	9.446
mmu-miR-322	3.247	up	4.672	6.371
mmu-miR-205	3.211	up	6.340	8.023
mmu-miR-362-5p	3.180	up	3.336	5.005
mmu-miR-671-5p	2.897	up	4.932	6.467
mmu-miR-702	2.712	up	3.336	4.776
mmu-miR-130b*	2.506	up	4.433	5.758
mmu-miR-1906	2.431	up	3.336	4.618
mmu-miR-670	2.386	up	8.183	9.438
mmu-miR-494	2.371	up	4.803	6.048
mmu-miR-680	2.346	up	5.070	6.300
mmu-miR-540-3p	2.225	up	4.420	5.574
mmu-miR-455*	2.172	up	3.336	4.455
mmu-miR-615-3p	2.051	up	3.336	4.372
mmu-miR-484	2.035	up	4.320	5.345
mmu-miR-34c	1.993	up	7.515	8.510
mmu-miR-323-3p	1.976	up	3.611	4.594
mmu-miR-101b	1.930	up	6.315	7.264
mmu-miR-216b	1.909	up	3.336	4.269
mmu-miR-1894-5p	1.822	up	3.336	4.202
mmu-miR-1224	1.809	up	8.638	9.493
mmu-miR-1904	1.773	up	7.139	7.965
mmu-miR-705	1.720	up	3.336	4.119
mmu-miR-770-3p	1.709	up	3.336	4.109
mmu-miR-485*	1.692	up	3.336	4.095
mmu-miR-152	1.663	up	9.775	10.509
mmu-miR-182	1.661	up	6.758	7.490
mmu-miR-337-3p	1.609	up	8.001	8.687
mmu-miR-433	1.576	up	5.812	6.468

mmu-miR-24-1*	14.853	down	11.075	7.182
mmu-miR-335-5p	8.754	down	6.466	3.336
mmu-miR-184	8.355	down	7.272	4.210
mmu-miR-29a*	6.029	down	5.928	3.336
mmu-miR-148a*	5.460	down	5.785	3.336
mmu-let-7b*	5.328	down	8.156	5.743
mmu-miR-100	4.378	down	7.677	5.546
mmu-miR-697	4.350	down	6.199	4.078
mmu-miR-199a-3p	3.436	down	5.526	3.745
mmu-miR-1198-5p	3.395	down	7.282	5.519
mmu-miR-30c-1*	3.168	down	5.000	3.336
mmu-miR-203	2.870	down	4.857	3.336
mmu-miR-676	2.699	down	8.424	6.991
mmu-miR-1187	2.652	down	5.113	3.706
mmu-miR-210	2.609	down	4.904	3.521
mmu-miR-30b*	2.579	down	7.401	6.035
mmu-miR-344b	2.441	down	5.952	4.665
mmu-miR-33	2.405	down	4.602	3.336
mmu-miR-28	2.235	down	8.202	7.042
mmu-miR-378	2.232	down	4.495	3.336
mmu-miR-32	2.223	down	4.489	3.336
mmu-miR-31*	2.205	down	4.477	3.336
mmu-miR-215	2.138	down	4.433	3.336
mmu-miR-539-5p	2.108	down	4.412	3.336
mmu-miR-301b	2.100	down	6.607	5.537
mmu-miR-223	2.071	down	5.285	4.235
mmu-miR-218	1.899	down	4.262	3.336
mmu-miR-10a	1.868	down	7.265	6.364
mmu-miR-1892	1.834	down	4.212	3.336
mmu-miR-700	1.825	down	4.204	3.336
mmu-miR-543	1.810	down	7.635	6.779
mmu-miR-541	1.762	down	7.147	6.330
mmu-miR-690	1.760	down	7.589	6.773
mmu-miR-384-5p	1.646	down	7.458	6.739
mmu-miR-328	1.632	down	7.343	6.636
mmu-miR-383	1.608	down	7.512	6.826
mmu-miR-202-3p	1.599	down	7.315	6.638
mmu-miR-181d	1.510	down	3.931	3.336

ESM FIGURE LEGENDS

ESM FIG 1. Up or downregulation of miRNA expression in rat islet and MIN6B1 cells. Dispersed rat pancreatic islets cells (A) or MIN6B1 cells (B) were transfected with the indicated miRNA mimics, anti-miRNAs or their respective controls (Ctrl or anti-ctrl). miRNA overexpression or downregulation was measured by qRT-PCR and normalized to U6 or miR-7 levels. Results are expressed as fold change *versus* Ctrl or as percentage of anti-ctrl and correspond to the mean ± SD of at least three independent experiments. * Significantly different from control (p-value ≤ 0.05, Student T-test).

ESM FIG. 2. Impact of specific miRNA changes on insulin content and secretion in MIN6B1 cells. MIN6B1 cells were transfected with the indicated miRNA mimics, anti-miRNAs or respective controls (Ctrl or anti-ctrl). Two days later, insulin secretion under basal (glucose 2 mmol/l, black bars) and stimulatory conditions (glucose 20 mmol/l, white bars) (A) and insulin contents (B) were determined. Insulin release is expressed as percentage of insulin content. The results represent means ± SD of four to five independent experiments. *Significantly different from control condition (p-value ≤ 0.05, ANOVA, Dunnett's post-hoc test).

ESM FIG 3. Impact of specific miRNA changes on MIN6B1 proliferation. MIN6B1 cells transfected with the indicated miRNA mimics (A) or anti-miRNAs (B) were stained with an antibody against Ki67 to assess cell proliferation. Prolactin (PRL 500 ng/ml during 48 h) and exendin-4 (100 nmol/l, 48h) were used as positive controls. The results are expressed as fold change *versus* the respective control and correspond to the mean ± SD of at least three independent experiments. * Significantly different from control condition (p-value ≤ 0.05, ANOVA, Dunnett's post-hoc test).

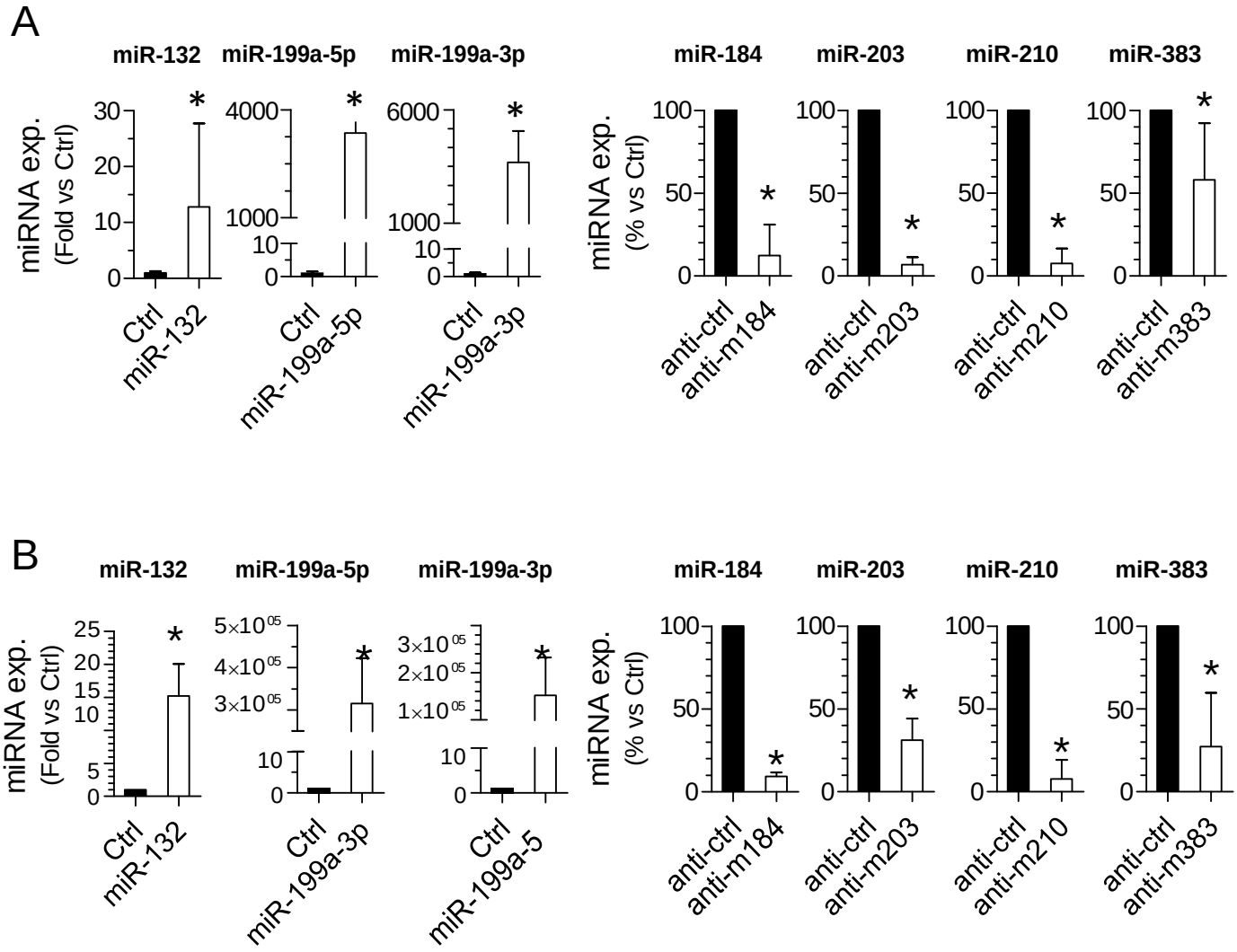
ESM FIG 4. Impact of specific miRNA changes on apoptosis in MIN6B1 cells. MIN6B1 cells were transfected with the indicated miRNA mimics, anti-miRNAs or the respective controls. Cell death was assessed two days later by determining the percentage of cells displaying pycnotic nuclei upon Hoechst staining. A mix of pro-inflammatory cytokines (IL-1β, TNFα and IFNγ) was used as a positive control for cell death. The results are expressed as means ± SD of at least three independent experiments. *Significantly different from control condition (p-value ≤ 0.05, ANOVA, Dunnett's post-hoc test).

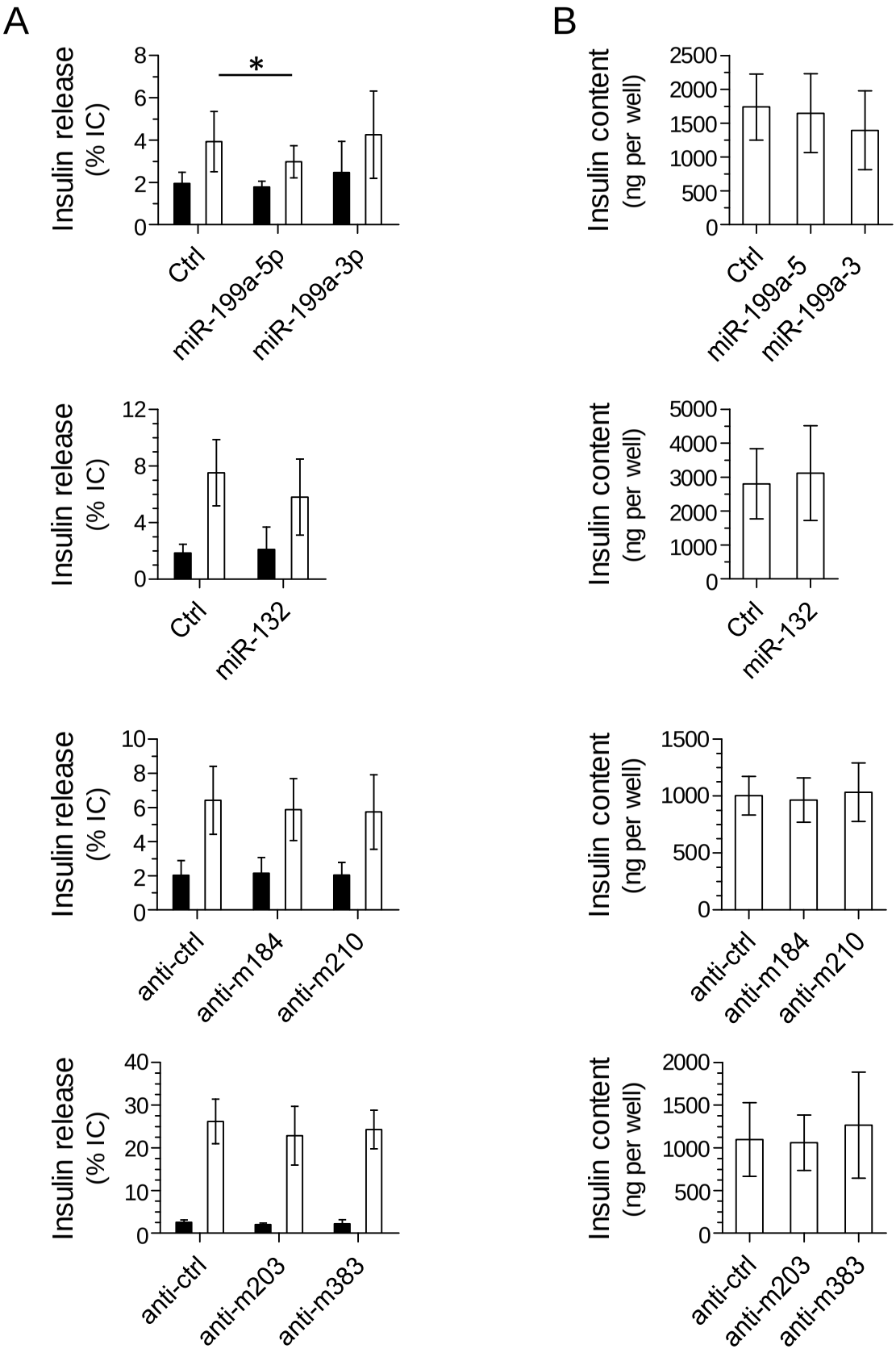
ESM FIG 5. Protective effect of miR-132 overexpression and miR-184 inhibition on palmitate- or cytokine-induced apoptosis. MIN6B1 cells transfected with miR-132 mimic, anti-miR-184 or the respective controls were exposed for 48 h with (white bars) or without (black bars) 0.5 mmol/l palmitate coupled to 0.5 % BSA (A) or for 24h with

(cross-hatched bars) or without (black bars) a mix of pro-inflammatory cytokines (B). Apoptosis was assessed 48 h post-transfection by counting the fraction of cells displaying pycnotic nuclei after Hoechst staining. The results correspond to the mean ± SD of four to six independent experiments. *Significantly different from control cells treated with the pro-apoptotic stimuli (p-value ≤ 0.05, ANOVA, Dunnett's post-hoc test).

ESM FIG 6. Potential targets of miR-199a-3p and miR-132. MIN6B1 cells were transfected with the indicated miRNA mimics. Two days later, the cells were homogenized and the lysates analyzed by Western blotting with antibodies against mTOR and cMET (A), GSK-3β (B) and granuphilin (C). The figure shows representative blots and band quantification from at least 3 independent experiments (means ± SEM). Protein levels were normalized to tubulin and expressed as fold change over control. *Significantly different from control condition (p-value ≤ 0.05 by ANOVA analysis and Dunnett's post-hoc test).

Nesca et al. ESM Fig 1

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B
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Insulin content (ng per well)
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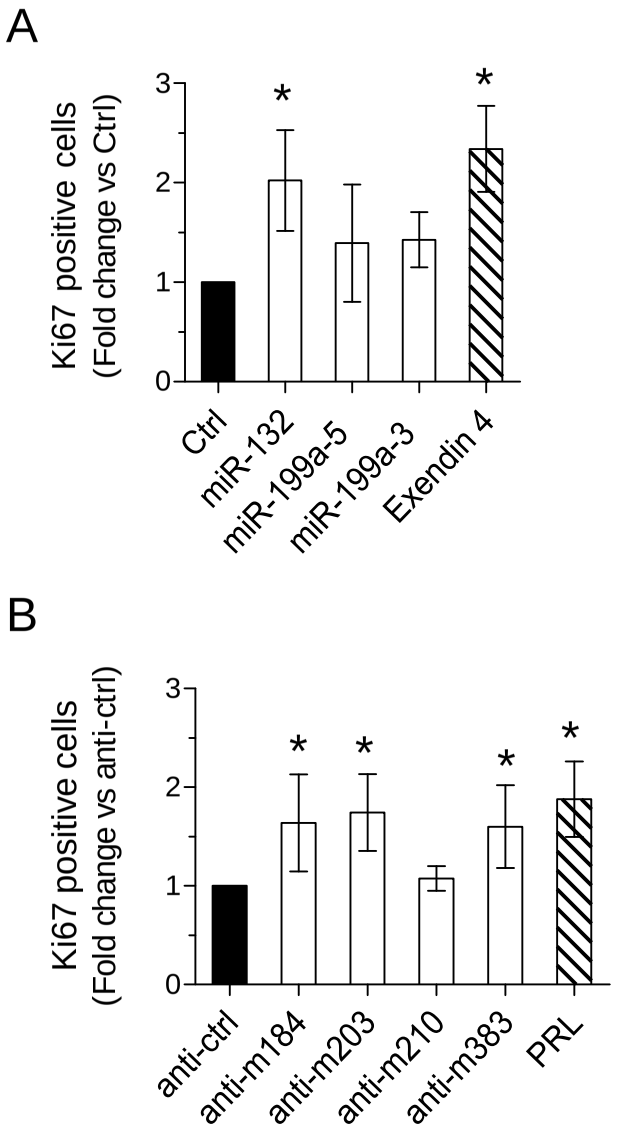
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Exendin 4
B
Ki67 positive cells
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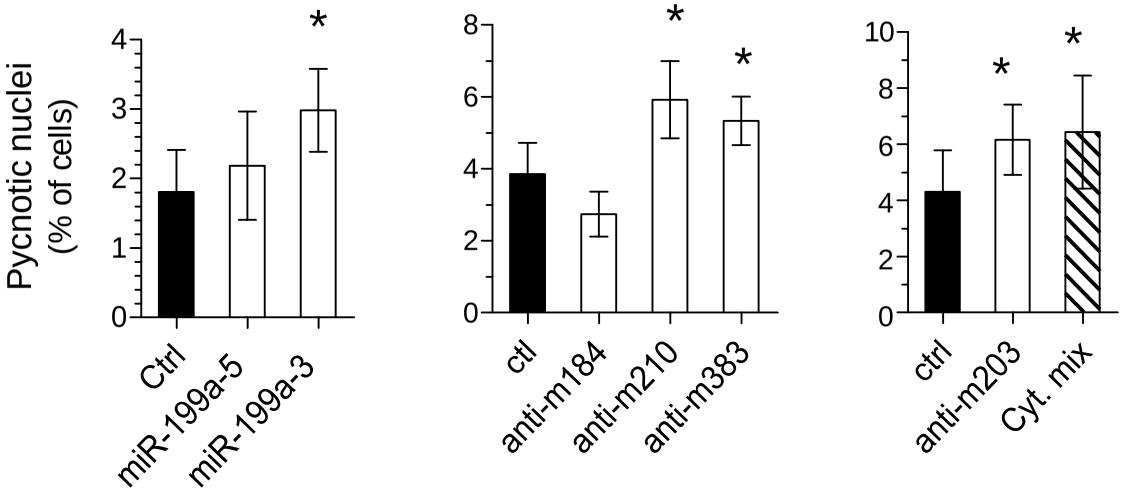
PRL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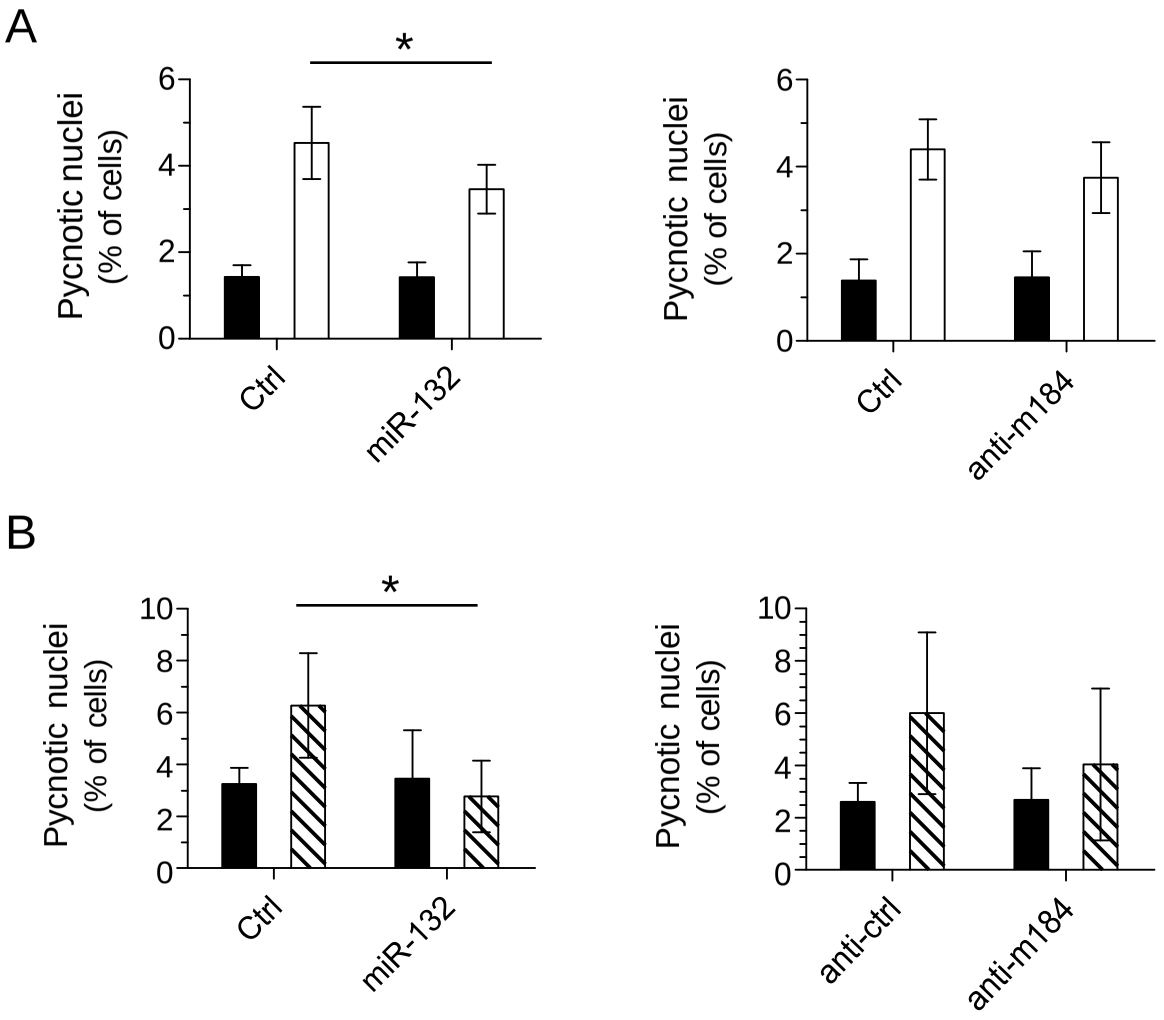
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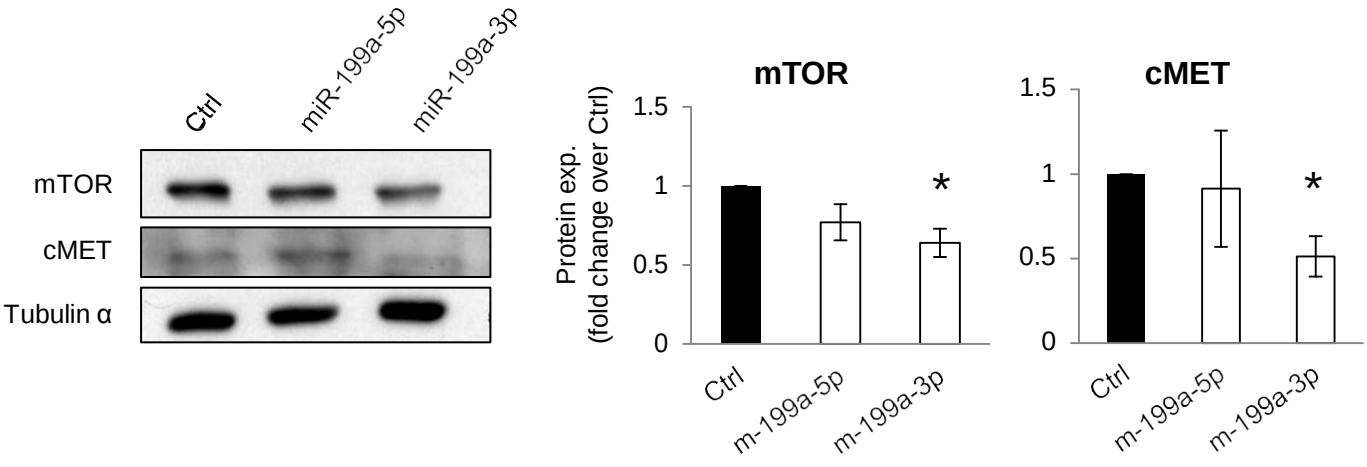
A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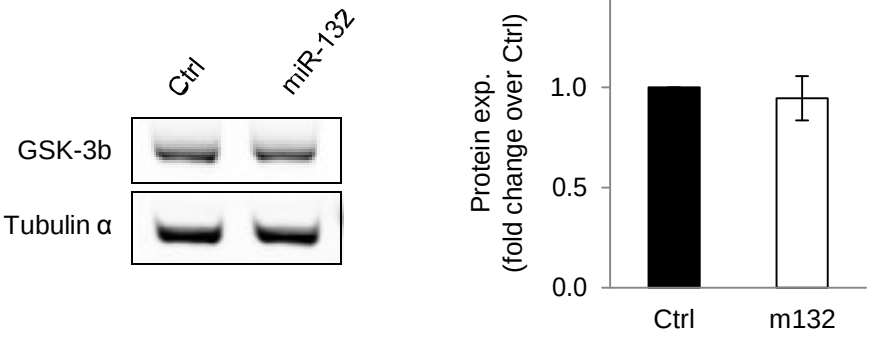
m-199a-5p
m-199a-3p

B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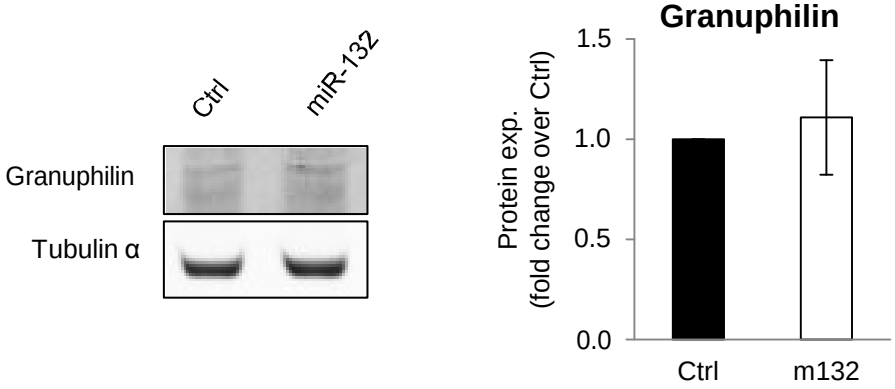
m132

C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