



miR-132-3p is a positive regulator of alpha-cell mass and is downregulated in obese hyperglycemic mice

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ABSTRACT

Objective: Diabetes is a complex disease implicating several organs and cell types. Within the islets, dysregulation occurs in both alpha- and beta-cells, leading to defects of insulin secretion and increased glucagon secretion. Dysregulation of alpha-cells is associated with transcriptome changes. We hypothesized that microRNAs (miRNAs) which are negative regulators of mRNA stability and translation could be involved in alpha-cell alterations or adaptations during type 2 diabetes.

Methods: miRNA microarray analyses were performed on pure alpha- and beta-cells from high-fat diet fed obese hyperglycemic mice and low-fat diet fed controls. Then, the most regulated miRNA was overexpressed or inhibited in primary culture of mouse and human alpha-cells to determine its molecular and functional impact.

Results: 16 miRNAs were significantly regulated in alpha-cells of obese hyperglycemic mice and 28 in beta-cells. miR-132-3p had the strongest regulation level in alpha-cells, where it was downregulated, while we observed an opposite upregulation in beta-cells. *In vitro* experiments showed that miR-132-3p, which is inversely regulated by somatostatin and cAMP, is a positive modulator of alpha-cell proliferation and implicated in their resistance to apoptosis. These effects are associated with the regulation of a series of genes, including proliferation and stress markers Mki67 and Bbc3 in mouse and human alpha-cells, potentially involved in miR-132-3p functions.

Conclusions: Downregulation of miR-132-3p in alpha-cells of obese diabetic mice may constitute a compensatory mechanism contributing to keep glucagon-producing cell number constant in diabetes.

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Keywords miR-132-3p; Alpha-cell; Glucagon; Proliferation; Apoptosis; Type 2 diabetes

1. INTRODUCTION

The control of glycemia depends largely on the coordinated secretions of glucagon and insulin by pancreatic alpha- and beta-cells. Hyperglycemia results when insulin-resistance is accompanied by dysfunction of pancreatic islet cells. The disrupted coordination of glucagon and insulin secretions observed in type 2 diabetes is characterized by impaired and delayed insulin secretion as well as basal hyperglucagonemia and non-suppressed glucagon secretion in response to glucose [1–3].

We generated obese, insulin-resistant, hyperglycemic mice using transgenic mice, expressing the fluorochrome Venus specifically in proglucagon-producing cells and the fluorochrome Cherry in insulin-producing cells, submitted to a high-fat diet (HFD) during 16 weeks. These mice have abnormal glucagon and insulin responses to glucose compared to control low-fat diet (LFD) mice, and specific molecular footprints including dysregulation of the *Gcg*, *Foxa1*, *cMaf*, *Pax6*, *UCP2*, and *Nav1.3* genes that are all involved in proglucagon gene expression

and glucagon secretion [4–8]. Furthermore, these cells express higher mRNA levels of *Pcsk1/3* and produce more GLP-1 than control alpha-cells, as previously reported, potentially explained by alpha-cell adaptation to hyperglycemia or to dedifferentiation [9,10].

miRNAs are small non-coding RNAs of 21–23 nucleotides [11]. They regulate the expression of target genes by inhibiting translation or by inducing mRNA degradation. After transcription, miRNA precursors are involved in a maturation process implicating the enzyme Drosha, exportin-5 and the endoribonuclease Dicer to form miRNAs [12].

Several studies, investigating Dicer function, have illustrated the importance of miRNAs in glucose homeostasis. Beta-cell-specific *Dicer1* knock-out mice, exhibit perturbed islet morphology, reduced beta-cell number, and altered differentiation as well as impaired GSIS resulting in progressive hyperglycemia and diabetes [13,14]. Beta-cell-specific knock-down of *Dicer1* in adult mice showed no impact on beta-cell architecture but strongly reduced insulin content and led to diabetes [15]. Multiple studies on islets or beta-cells have identified miRNAs implicated in beta-cell function, such as miR-375 and miR-

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124a, and beta-cell compensation or failure, such as miR-132, miR-184 or miR-338-3p [16,17]. Most of these miRNAs were shown to be up- or downregulated in different diabetic rodent models and human islets [18,19]. Moreover, it appears that clusters of miRNAs can be co-regulated by common mechanisms such as transcription factors or epigenetic regulators [19,20].

Although most of the studies focused on beta-cells, miRNAs are also important in alpha-cell biology and function. A deletion of *Dicer1* in the endocrine pancreas using Cre under the control of the *Pdx1* promoter resulted in a gross defect in the endocrine lineage including severe reductions of alpha-cell number [21]. Moreover, deletion of miR-375 in mice induced an increase of alpha-cell number, of fasting and fed glucagon plasma levels, and of gluconeogenesis and hepatic glucose output [22]. We hypothesized that miRNAs are involved in alpha-cell molecular and functional alterations/adaptations in type 2 diabetes. In the present study, we identified 16 miRNAs differentially regulated in alpha-cells and 28 in beta-cells isolated from hyperglycemic HFD compared to LFD mice. miRNAs regulated in alpha-cells were studied *in vitro* in mouse primary alpha-cells to investigate their regulation and biological functions. We now report that the most highly differentially regulated miRNA in alpha-cells from hyperglycemic HFD mice, miR-132-3p, is involved in alpha-cell proliferation and survival, is inversely regulated by somatostatin and cAMP and potentially regulates alpha-cell number during diabetes.

2. MATERIAL AND METHODS

2.1. Animals

The GLU-Venus x INS-Cherry mice express the Venus and Cherry fluorochromes respectively in proglucagon- and insulin-producing cells [8,23]. Mice were bred in conventional housing with a 12/12 h dark/light period (lights on at 7:00 AM) at the University of Geneva Medical School animal facility, according to ethical approbation by the Swiss federal committee.

Mice used to generate alpha- and beta-cells included in the miRNA microarray experiments were fed by either a LFD containing 10% kcal fat (D12450B, Research Diets) (protein: 20% kcal; carbohydrate: 70% kcal; energy density 3.82 kcal/g), or a HFD containing 60% kcal fat (D12492, Research Diets) (protein: 20% kcal; carbohydrate: 20% kcal; energy density: 5.21 kcal/g), from the age of 10 weeks and for 16 weeks. At the end of 16 weeks of HFD feeding, mice which did not develop obesity (weight < 40 g) or hyperglycemia (HbA1c < 4.5%), evaluated by Siemens DCA systems Hemoglobin A1c (Siemens Healthcare Diagnostics Inc.) were excluded from the study (Supp Table A).

Mice used to generate primary cells for *in vitro* experiments were fed a normal chow diet until sacrifice.

2.2. Mouse alpha- and beta-cell sorting and primary culture

Venus⁺ mouse alpha-cells and Cherry⁺ beta-cells were prepared by fluorescence-activated cell sorting (FACS) using Biorad S3 and Beckman Coulter Astrios, after islet isolation as previously described [24]. FACS-purified alpha- and beta-cells used for microarray and validation qPCR analyses were collected and stored at -80 °C until RNA extraction. Mouse alpha-cells used for primary culture were placed in 10% FBS supplemented DMEM with 5.6 mM glucose for overnight recovery and, then, seeded on 804G matrix. For miRNA mimicking or inhibition experiments, optimal conditions were determined by settings prior to the experiments. The cells were transfected 24 h after plating with 100 nM miR-132-3p mirVana mimics and

inhibitors or respective negative controls (Ambion) using Lipofectamine RNAiMax (Invitrogen). 48 h after transfection, miR-132-3p had a 128.7 fold overexpression and a 9.5 fold downregulation respectively (Supp Fig. A). For *in vitro* regulation of miRNA expression experiments, mouse alpha-cells were placed, 24 h after plating, for 48 h in 1% FBS supplemented DMEM with 0.5% BSA and glucose, palmitate, insulin, IBMX + forskolin or somatostatin at different concentrations.

2.3. Human alpha-cell sorting and primary culture

Human islets from non-diabetic donors were obtained from Prodo Laboratories (Prodo Laboratories Inc, Aliso Viejo, CA). Twenty-four hours after delivery, 2000 islets (IEQ) were dissociated using trypsin treatment (5 min at 37 °C) with pipetting every minutes. Dissociated islets cells were then labeled with anti-human alpha-cell antibody HPa1 (DHIC2-2C12, Thermo Scientific; 1/50, 30 min at 4 °C) and Alexa Fluor 488 goat anti mouse IgM (Life Technologies; 1/200, 20 min at 4 °C) as previously described [25]. Dead cells were marked with DRAQ7 (Far-Red Fluorescent Live-Cell Impermeant DNA Dye, Biostatus). Human alpha-cell fractions were collected with exclusion of dead cells and doublets using BioRad S3 cell sorter as described [26]. Primary human alpha-cells were then cultured in PIM(S) medium using standard procedures [8] and transfected 24 h after plating with 100 nM miR-132-3p mirVana mimics and inhibitors or respective negative controls (Ambion) using Lipofectamine RNAiMax (Invitrogen).

2.4. Total RNA and mRNA extraction

mRNA and total RNA, containing miRNAs, were isolated from mouse pancreatic alpha- and beta-cells as well as human alpha-cells with RNeasy plus and miRNeasy micro kits (Qiagen) following manufacturer instructions.

2.5. miRNA microarray

Alpha-cell total RNA extracts from 2 to 3 mice were pooled to obtain 100 ng of total RNA for each sample. The samples were labeled according to the Agilent miRNA Complete Labeling and Hybridization Kit. The labeled RNAs were hybridized to Agilent Mouse miRNA Microarray (Release 21.0, 8 × 60 K; Catalog number G4872A-070155; Agilent Technologies) with 1,881 mouse miRNAs represented, for 20 h at 55 °C with rotation. After hybridization and washing, the arrays were scanned with an Agilent microarray scanner using high dynamic range settings as specified by the manufacturer. Agilent Feature Extraction Software v10.7.3.1 was used to extract the data. Samples were then quantile normalized based on summarized and log₂-transformed expression levels of probe sets. Differentially expressed miRNAs were determined using a moderated t-test implemented in the R Bioconductor package limma. Five contrasts were extracted from the linear model: alpha-cells (HFD) vs alpha-cells (LFD); beta-cells (HFD) vs beta-cells (LFD); alpha-cells (HFD) vs beta-cells (HFD); alpha-cells (LFD) vs beta-cells (LFD); the interaction between cell type and diet type. In order to correct for multiple testing, p-values were adjusted across all five contrasts by the Benjamini-Hochberg method, which controls for false discovery rate.

2.6. miRNA targets and pathways computational analysis

Predicted targets of miRNAs were determined with the miRSystem tool (<http://mirsystem.cgm.ntu.edu.tw>). Were only considered validated targets or targets predicted by, at least, three different algorithms. KEGG pathways analysis was performed with DIANA-miRPath v.3 pathway analysis web-server [27].

2.7. Glucagon secretion tests

48 h after transfection, primary mouse alpha-cells were incubated in Krebs buffer containing 5.6 mM glucose for 2 h and then successively in Krebs buffer containing 5.6 mM glucose for 30 min (basal) and 5.6 mM glucose + 10 mM arginine or 1 mM glucose for 30 min (stimulated). Supernatants were collected at the end of each 30 min incubation step and cells were lysed in acid/ethanol mixture at the end of tests for glucagon cell content measurements. Glucagon was quantified with specific ELISA kits for mature glucagon (Mercodia AB).

2.8. Proliferation and apoptosis measurements

Primary mouse alpha-cell proliferation was evaluated by BrdU incorporation for 48 h after transfection of miRNA mimics and inhibitors. BrdU⁺ cells were labeled with 5-Bromo-2'-deoxy-uridine Labeling and detection Kit I (Roche). Alpha-cell apoptosis was evaluated 48 h after transfection by TUNEL staining with In Situ Cell Death Detection Kit, TMR red (Roche) or immuno-labeling with anti-active Caspase-3 primary antibody (1:25) (ab2302, abcam) and Alexa Fluor 568 antirabbit (1:500) secondary antibody. Co-staining for glucagon with rabbit anti-glucagon (1:500; Millipore Corporation) primary antibodies and Alexa Fluor 488 antirabbit (1:1,000) secondary antibodies was realized in parallel to BrdU or TUNEL and active Caspase-3 staining. At least 35 independent fields in each condition were captured with an Evos FL fluorescence microscope (Life Technologies) and automatically analyzed with Metamorph software to determine apoptotic and proliferative rates.

2.9. mRNAs and miRNAs expression qPCR-quantification

After reverse transcription of mRNAs (Prime-script RT Reagent, Takara Bio Inc.), cDNA were pre-amplified (cDNA Pre-Amp Master, Roche Diagnostics) and analyzed by real-time quantitative PCR (qPCR) using LightCycler 480 SYBR Green technology (Roche Diagnostics) as previously described [8,24].

miRNAs were reverse-transcribed and pre-amplified (14 cycles) with the Taqman Advanced miRNA Assays technology (Applied Biosystems) according to manufacturer instructions. The expression of miRNAs was then quantified by qPCR with the Taqman Fast Advanced technology (Applied Biosystems) with a LightCycler 480 (Roche) and normalized to the expression of housekeeping miRNAs (miR-186-5p; miR-361-5p) exhibiting stable expression across tested condition in our experiments (Supp Fig. B).

2.10. Data analyses

Paired and unpaired one-tailed and two-tailed t-tests as well as multiple t-test with Benjamini, Krieger and Yekutieli method and two-way ANOVA with Tukey's multiple comparison post-hoc test were performed with Prism GraphPad-software (v7.02) when appropriate. Data are presented as means \pm SEM and are significant at $P \leq 0.05$ or FDR ≤ 0.05 .

3. RESULTS

3.1. Regulation of miRNAs in alpha- and beta-cells of obese hyperglycemic mice

We performed microarray analyses on 5 LFD and 6 HFD FACS-sorted mice alpha-cell samples and on 6 LFD and 6 HFD FACS-sorted mice beta-cell samples. Statistical analysis indicated that 125 miRNAs were differentially expressed in alpha- versus beta-cells in the LFD group (Supp Table B) and 150 in alpha-cells compared to beta-cells from HFD mice (Supp Table C). 16 miRNAs were significantly differentially regulated in alpha-cells from HFD compared to LFD mice. Among these

16 miRNAs, 14 were downregulated and 2 were upregulated (Table 1). 28 miRNAs were significantly differentially regulated in beta-cells from HFD vs LFD mice; 6 were downregulated and 22 upregulated (Table 2). In alpha-cells, the most differentially regulated miRNA in obese hyperglycemic mice compared to controls was miR-132-3p with a 2.26 fold decrease. miR-132-3p was, by contrast, upregulated in beta-cells with a 1.67 fold increase. An interaction between cell types and metabolic status was found for 14 miRNAs, including miR-132-3p, indicating that changes of these miRNAs by HFD-feeding were influenced by cell types (Supp Table D). miR-132-3p was also differentially expressed in alpha- versus beta-cells with 5.84 and 21.9 fold enrichments in beta-cells of LFD and HFD mice respectively (Figure 1A), indicating that miR-132-3p is much more abundant in beta- than in alpha-cells and is inversely regulated in these cells during diabetes. We measured by qPCR the expression of miR-132-3p in FACS-sorted alpha-cells from 9 LFD and 7 HFD mice and confirmed the downregulation of this miRNA with a 2.8 fold decrease in obese hyperglycemic HFD compared to LFD mice (Figure 1B).

3.2. Downregulation of the *Meg3-Mirg* cluster in hyperglycemic mice

Interestingly, 8 of the 14 significantly downregulated miRNAs in alpha-cells of obese hyperglycemic mice, miR-487b-3p; miR-369-5p; miR-382-5p; miR-409-3p; miR-431-5p; miR-127-3p; miR-329-3p; miR-382-3p, were located in a common genomic cluster, the *Meg3-Mirg* cluster, expressed from the maternally inherited chromosome 12 (Supp Fig. C). Suggesting that the whole cluster is downregulated in alpha-cells of diabetic mice.

3.3. miRNAs potential targets

The potential functions associated to the miRNAs differentially regulated in alpha-cells from obese hyperglycemic mice were investigated by computational analyses. A miRNA KEGG pathway analysis performed with DIANA-miRPath predicted significant interactions with 11 pathways, including several pathways implicated in lipid metabolism (fatty acid biosynthesis, fatty acid metabolism,

Table 1 — Regulated alpha-cell miRNAs in HFD obese hyperglycemic vs LFD mice.

ID	logFC	FoldChange	mean alpha HFD	mean alpha LFD	adj.P.Val
mmu-miR-132-3p	-1.18	-2.26	1.28	2.45	6.92E-04
mmu-miR-7686-5p	0.94	1.92	3.44	2.50	3.65E-02
mmu-miR-6937-5p	0.79	1.73	4.13	3.35	3.46E-02
mmu-miR-487b-3p	-0.70	-1.62	2.56	3.26	2.20E-04
mmu-miR-103-3p	-0.44	-1.36	7.43	7.88	2.50E-03
mmu-miR-431-5p	-0.42	-1.34	3.08	3.50	2.30E-02
mmu-miR-369-5p	-0.40	-1.32	2.18	2.58	1.16E-02
mmu-miR-382-3p	-0.38	-1.30	2.62	3.01	3.75E-02
mmu-miR-409-3p	-0.37	-1.30	3.93	4.30	1.90E-02
mmu-miR-99b-5p	-0.37	-1.29	4.16	4.54	2.42E-02
mmu-miR-652-3p	-0.34	-1.27	6.07	6.41	2.42E-02
mmu-miR-382-5p	-0.34	-1.26	4.69	5.03	1.74E-02
mmu-miR-129-5p	-0.33	-1.26	6.47	6.80	1.52E-02
mmu-miR-127-3p	-0.32	-1.25	7.75	8.07	2.73E-02
mmu-miR-329-3p	-0.31	-1.24	4.28	4.59	3.42E-02
mmu-miR-107-3p	-0.30	-1.23	7.45	7.75	3.51E-02

Identification by microarray analyses of miRNAs differentially expressed in FACS sorted HFD mouse alpha-cells (n = 6) versus FACS sorted LFD mouse alpha-cells (n = 5). The 16 significantly differentially expressed miRNAs are presented and ranked by fold change absolute value.

Table 2 — Regulated beta-cell miRNAs in HFD obese hyperglycemic vs LFD mice.

ID	logFC	FoldChange	mean_beta_HFD	mean_beta_LFD	adj.P.Val
mmu-miR-184-3p	-3.05	-8.29	2.51	5.57	6.63E-06
mmu-miR-146a-5p	1.06	2.09	1.17	0.11	6.92E-03
mmu-miR-212-3p	0.99	1.98	2.50	1.52	1.26E-03
mmu-miR-141-5p	0.93	1.90	2.86	1.93	4.27E-02
mmu-miR-138-5p	0.92	1.89	2.02	1.11	1.52E-02
mmu-miR-130b-3p	0.88	1.84	5.39	4.51	3.27E-05
mmu-miR-132-3p	0.74	1.67	5.73	4.99	1.32E-02
mmu-miR-344b-3p	0.68	1.60	2.46	1.78	3.40E-02
mmu-miR-152-3p	0.63	1.55	7.35	6.72	1.74E-03
mmu-miR-15b-5p	0.63	1.54	6.06	5.43	6.95E-04
mmu-miR-574-3p	0.59	1.51	2.21	1.62	2.41E-02
mmu-miR-362-3p	0.53	1.45	3.35	2.82	2.73E-02
mmu-miR-1895	-0.52	-1.43	3.11	3.63	7.42E-03
mmu-miR-7082-5p	-0.49	-1.41	2.76	3.26	1.91E-02
mmu-miR-676-3p	0.49	1.41	2.61	2.12	4.44E-02
mmu-miR-182-5p	0.47	1.38	2.55	2.09	1.46E-02
mmu-miR-27a-3p	0.45	1.37	5.16	4.70	1.54E-02
mmu-miR-23b-3p	-0.45	-1.36	7.42	7.87	7.42E-03
mmu-miR-320-3p	0.44	1.36	3.22	2.77	2.94E-02
mmu-miR-106b-5p	0.43	1.35	4.73	4.29	1.20E-02
mmu-miR-141-3p	0.42	1.33	8.41	8.00	2.94E-02
mmu-miR-148a-3p	0.41	1.33	10.06	9.65	4.57E-02
mmu-miR-15a-5p	0.39	1.31	7.78	7.39	1.84E-02
mmu-miR-21a-5p	0.37	1.29	6.06	5.69	3.74E-02
mmu-miR-22-3p	0.36	1.29	8.83	8.47	2.52E-02
mmu-miR-200a-3p	0.36	1.28	8.39	8.03	1.45E-02
mmu-miR-652-3p	-0.32	-1.25	6.89	7.21	2.47E-02
mmu-miR-103-3p	-0.30	-1.23	7.06	7.36	2.77E-02

Identification by microarray analyses of miRNAs differentially expressed in FACS sorted HFD mouse beta-cells (n = 6) versus FACS sorted LFD mouse beta-cells (n = 6). The 28 significantly differentially expressed miRNAs are presented and ranked by fold change absolute value.

fatty acid degradation, steroid biosynthesis) as well as amino acids metabolism (tryptophan metabolism, lysine degradation, lysine biosynthesis) (Table 3).

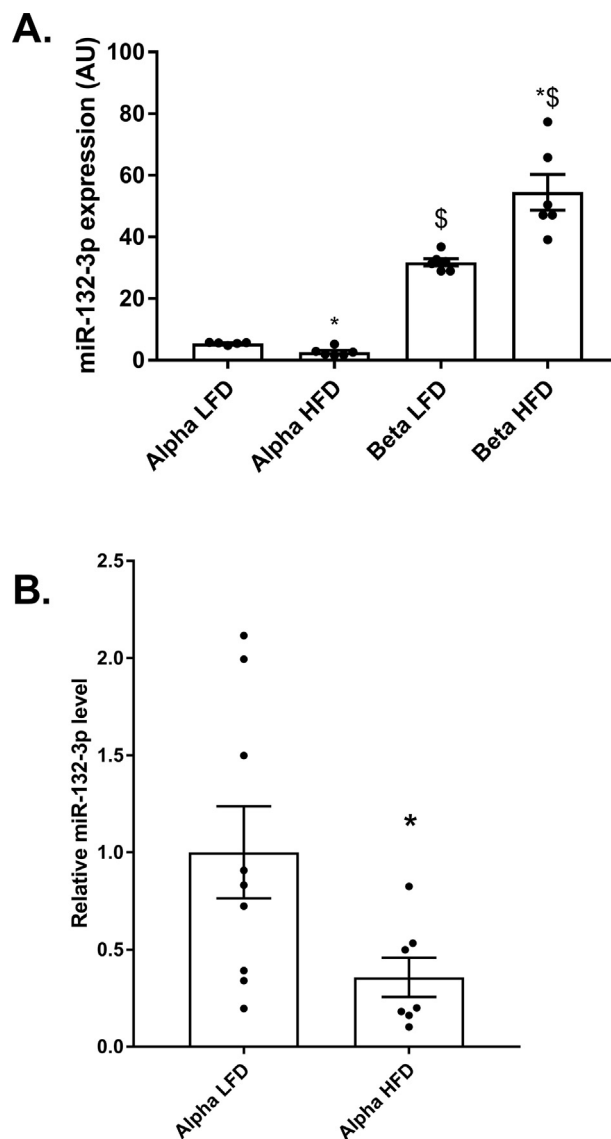


Figure 1: miR-132-3p expression in alpha- and beta-cells in LFD and HFD mice. (A) Level of expression in the microarray analyses of miR-132-3p in FACS sorted alpha-cells from LFD mice (n = 5), HFD mice (n = 6), and FACS sorted beta-cells from LFD mice (n = 6) and HFD mice (n = 6). Moderated t-test, Benjamini-Hochberg method * Adj.P.Val. ≤ 0.05 vs LFD; \$ Adj.P.Val. ≤ 0.05 vs Alpha-cells. (B) Expression level of miR-132-3p was measured by qPCR in FACS sorted alpha-cells from LFD mice (n = 9) and HFD mice (n = 7). Two-tailed unpaired Student's t test, *P ≤ 0.05 vs LFD.

Predicted targets of the 16 miRNAs regulated in alpha-cells from obese hyperglycemic mice were determined by the miRSystem tool and compared to the mRNAs previously described as regulated in diabetic alpha-cells [8]. The miRNA showing the most hits was miR-132-3p with four predicted targets belonging to the diabetic alpha-cell molecular footprint (*Foxa1*, *cMaf*, *Isl1* and *Sca3a*). *Isl1*, *UCP2* and *Sca3a* were also predicted targets of miR-652-3p, miR-431-5p and miR-409-3p respectively (Supp Table E).

3.4. Regulation of miR-132-3p expression *in vitro*

We compared the sequences of mouse, rat and Human mir-132. mir-132 is highly conserved between these species. Sequences of the mouse and rat precursors are identical and have only one base change with the human sequence (Supp Fig. D.1–2). The 22 nucleotides

Table 3 — Computational analyses of miRNAs putative pathways.

KEGG pathway	p-value	#genes	#miRNAs
Fatty acid metabolism	1.62E-22	8	3
Fatty acid biosynthesis	1.67E-22	1	2
Adherens junction	3.99E-09	15	7
Fatty acid degradation	6.37E-09	7	3
Tryptophan metabolism	4.90E-05	8	3
Lysine degradation	1.77E-04	9	5
Proteoglycans in cancer	1.99E-03	24	6
Steroid biosynthesis	2.67E-03	2	2
Renal cell carcinoma	6.14E-03	11	3
Protein processing in endoplasmic reticulum	2.27E-02	22	8
Lysine biosynthesis	2.32E-02	1	2

KEGG pathway analyses, on the 16 miRNAs significantly differentially expressed in FACS sorted HFD mouse alpha-cells versus FACS sorted LFD mouse alpha-cells, performed with DIANA-miRPath v.3 pathway analysis web-server.

sequence of miR-132-3p mature form is identical in the three species (Supp Fig. D.3).

In order to identify which factors are responsible for the regulation of miR-132-3p in alpha-cells of obese hyperglycemic mice, we treated *in vitro* primary mouse alpha-cells with different factors mimicking hyperlipidemia, hyperglycemia, or hyperinsulinemia occurring in obesity and/or diabetes and measured miR-132-3p expression by qPCR. The expression of miR-132-3p was not significantly regulated after 48 h treatment with palmitate 500 μ M plus glucose 25 mM, palmitate 500 μ M alone, glucose 25 mM alone, nor by insulin 100 nM (Figure 2A). As cAMP was reported to be strongly implicated in miR-132-3p regulation in beta-cells [20,28], we treated primary mouse alpha-cells with IBMX 100 μ M and forskolin 50 μ M during 48 h. This treatment increased by 45% the expression of miR-132-3p, indicating that cAMP is a positive regulator of miR-132-3p expression (Figure 2B). Finally, we evaluated the impact of somatostatin, a potential modulator of cAMP through its coupling with

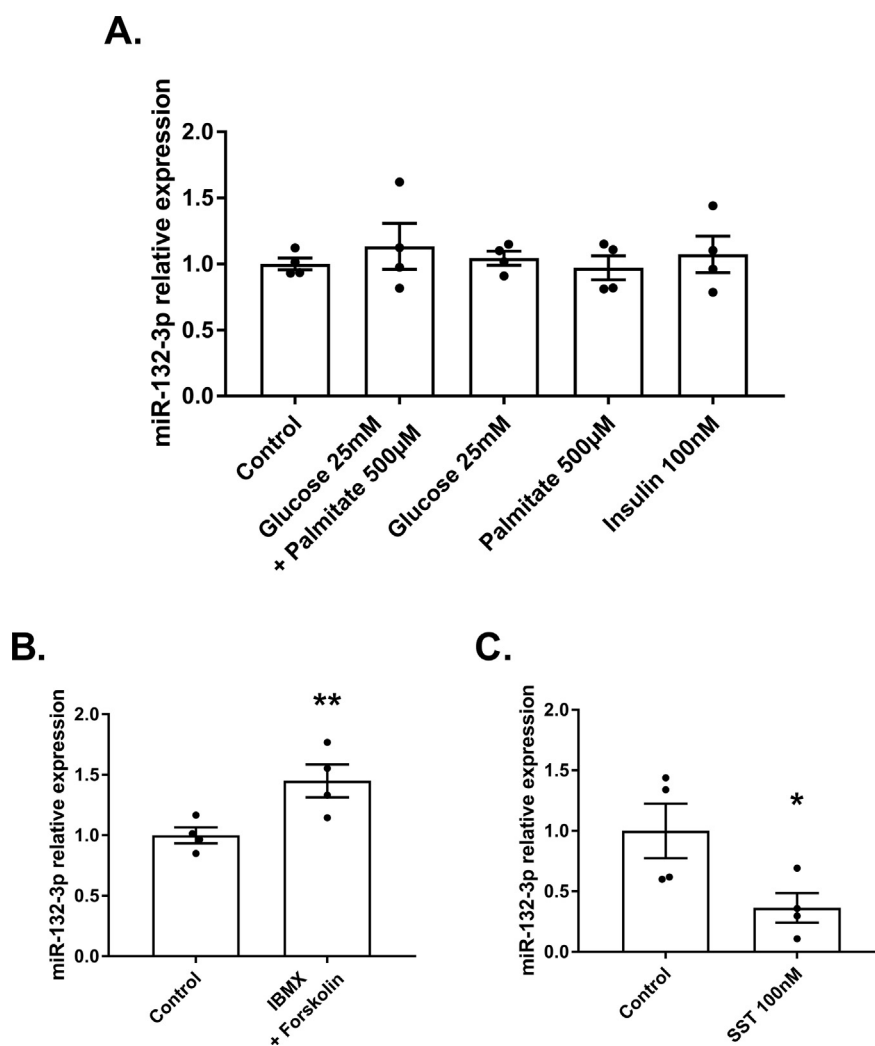


Figure 2: Regulation of miR-132-3p in primary cultures of mouse alpha-cells. (A) Evaluation by qPCR of the expression of miR-132-3p in primary alpha-cells cultured for 48 h in 1% FBS supplemented DMEM with 0.5% BSA and 5.6 mM glucose (Control), 25 mM glucose and 500 μ M palmitate, 25 mM glucose, 500 μ M palmitate or 100 nM insulin (n = 4). (B) Evaluation by qPCR of the expression of miR-132-3p in primary alpha-cells cultured in 1% FBS supplemented DMEM with 0.5% BSA and 5.6 mM glucose with control or 10 μ M IBMX + 10 μ M Forskolin treatment during 48 h (n = 4). (C) Evaluation by qPCR of the expression of miR-132-3p in primary alpha-cells cultured in 1% FBS supplemented DMEM with 0.5% BSA and 5.6 mM glucose with control or 100 nM somatostatin treatment during 48 h (n = 4). One-tailed paired Student's t test, *P \leq 0.05, **P \leq 0.01 vs control.

$G_{i/o}$ pathway, on miR-132-3p expression. Somatostatin at 100 nM during 48 h induced a 2.7 fold downregulation of miR-132-3p, in line with the regulation observed *in vivo* in obese hyperglycemic mice (Figure 2C).

3.5. Functional impact of miR-132-3p in primary mouse alpha-cells

In order to investigate the potential consequences of miR-132-3p decreases observed in alpha-cells of obese hyperglycemic mice, we treated primary mouse alpha-cells for 48 h with miR-132-3p inhibitors and mimics and performed glucagon secretion tests in basal and stimulated conditions with arginine or low glucose. miR-132-3p mimicking or inhibition affected neither basal nor arginine- and low glucose-stimulated glucagon secretions (Figure 3A,B). Modulating miR-132-3p levels did not change the glucagon secretion fold-change between basal and stimulated conditions, indicating that miR-132-3p is not implicated in the stimulation of glucagon secretion by arginine or low glucose (Figure 3A,B).

As, on one hand, the literature shows that miR-132 is a positive regulator of proliferation in beta-cells [29] and that alpha-cell mass can

vary during diabetes [30,31]; and as on the other hand, we observed that alpha-cell mass is unchanged at the end of 16 weeks of HFD protocol [8], while miR-132-3p is downregulated, we postulated that the miR-132-3p decrease in alpha-cells may be involved in the control of alpha-cell mass. We explored the impact of miR-132-3p inhibition and mimicking on primary mouse alpha-cell by BrdU incorporation. 48 h after transfection with miR-132-3p mimic, we observed a 47.7% increase of BrdU incorporation and a 21.2% decrease 48 h after transfection with the inhibitor, indicating that miR-132-3p is able to promote alpha-cell proliferation, and thus alpha-cell number (Figure 4A,B). Alpha-cells are also characterized by a high resistance to metabolic stress-induced apoptosis [32]. We hypothesized that miR-132-3p could be involved in this process and performed TUNEL and active Caspase-3 staining, 48 h after transfection with miR-132-3p mimic or inhibitor in standard and glucolipotoxicity culture conditions. miR-132-3p mimic reduced alpha-cell apoptosis through by a non-significant 21% decrease of TUNEL positive cells and a 37% significant decrease of active Caspase-3 positive cells in standard conditions (Figure 4C,E). Inversely, miR-132-3p inhibition induced an increase by 79% and by 66% of TUNEL and active Caspase-3

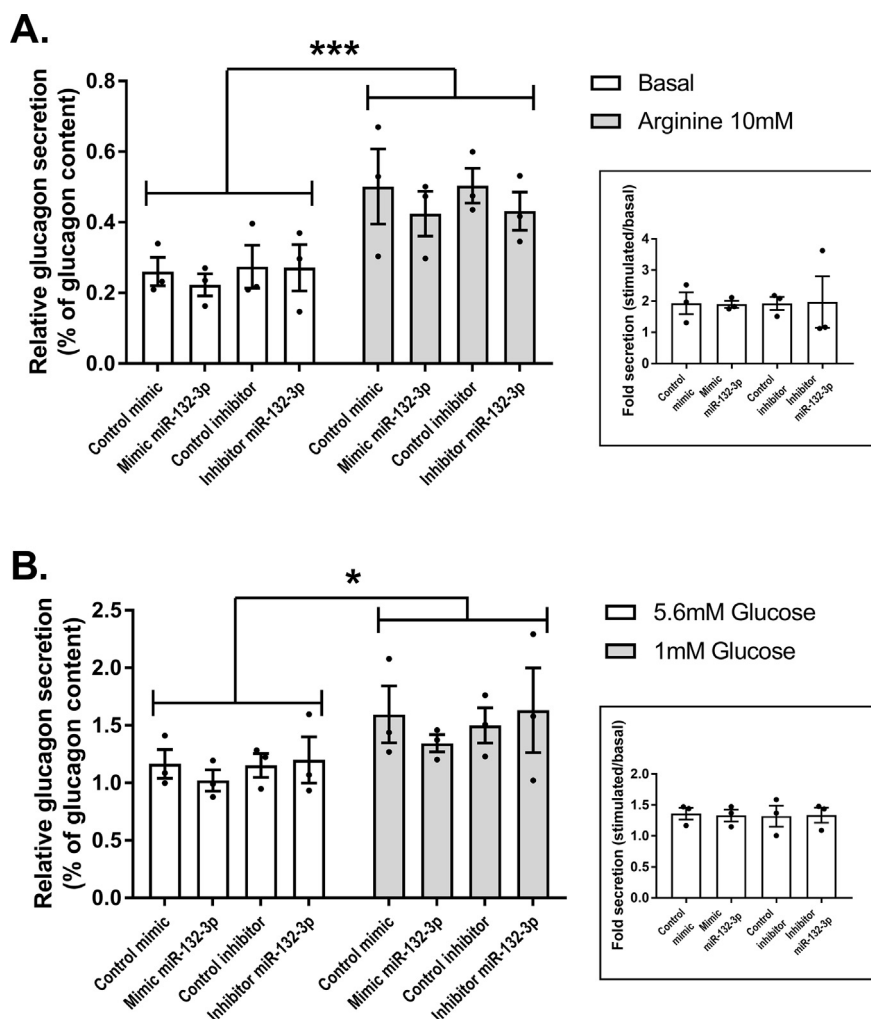


Figure 3: Impact of miR-132-3p on glucagon secretion. (A) 5.6 mM glucose basal and 10 mM arginine stimulated glucagon secretion during 30 min from primary mouse alpha-cells 48 h after transfection with 100 nM of miR-132-3p mimic or inhibitor expressed in % of total glucagon cell content (n = 3) or in fold (stimulated/basal) (insert) (n = 3). (B) 5.6 mM glucose basal and 1 mM glucose stimulated glucagon secretion during 30 min by primary mouse alpha-cells 48 h after transfection with 100 nM of miR-132-3p mimic or inhibitor expressed in % of total glucagon cell content (n = 3) or in fold (stimulated/basal) (insert) (n = 3). Two-way ANOVA, *P ≤ 0.05, ***P ≤ 0.001 vs 5.6 mM glucose basal.

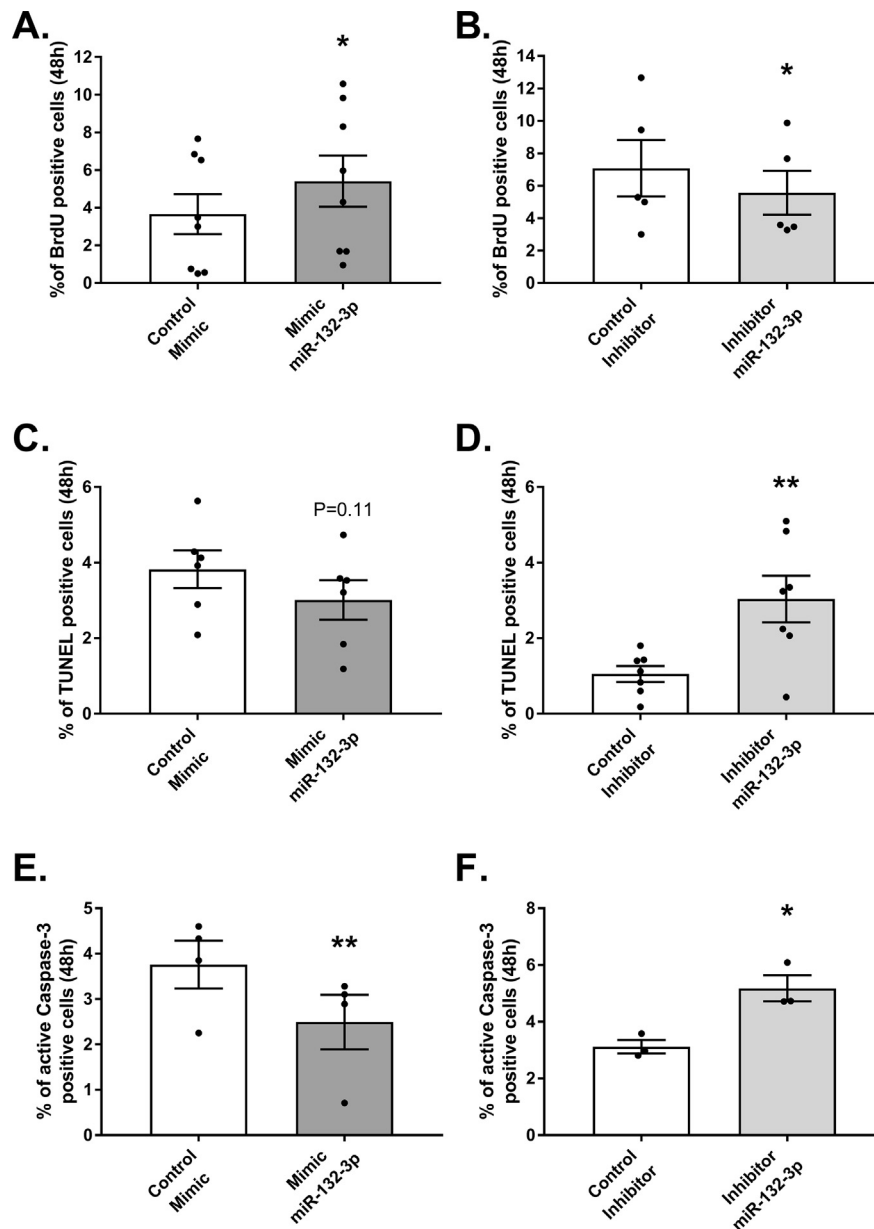


Figure 4: Impact of miR-132-3p on alpha-cell proliferation and apoptosis. (A–B) 48 h BrdU incorporation in primary mouse alpha-cells transfected with 100 nM control mimic or inhibitor (white) or miR-132-3p mimic (dark grey) (n = 8) (A) or inhibitor (light grey) (n = 5) (B). **(C–D)** TUNEL labeling in primary mouse alpha-cells transfected with 100 nM control mimic or inhibitor (white bars) or miR-132-3p mimic (dark grey) (n = 6) (C) or inhibitor (light grey) (n = 6) (D) and cultured in 1% FBS supplemented DMEM with 0.5% BSA and 5.6 mM glucose for 48 h. **(E–F)** Active Caspase-3 labeling in primary mouse alpha-cells transfected with 100 nM control mimic or inhibitor (white bars) or miR-132-3p mimic (dark grey) (n = 4) (E) or inhibitor (light grey) (n = 3) (F) and cultured in 1% FBS supplemented DMEM with 0.5% BSA and 5.6 mM glucose for 48 h. Two-tailed paired Student's t test, *P ≤ 0.05, **P ≤ 0.01 vs control.

positive cells respectively in standard culture conditions (Figure 4D,F). In glucolipotoxicity conditions TUNEL positive cells were reduced by miR-132-3p mimicking and increased by its inhibition while active Caspase 3 staining was not significantly affected (Supp Fig. E). These results indicate that miR-132-3p is implicated in the resistance of alpha-cells to apoptosis *per se* in control or metabolic stress conditions.

3.6. Molecular impact of miR-132-3p

To better understand and characterize the mechanisms underlying the miR-132-3p functions in alpha-cells, we measured by qPCR the

expression of genes coding for proteins involved in alpha-cell identity, glucagon production, cell proliferation and cellular stress response, 48 h after transfection with miR-132-3p mimic or inhibitor. miR-132-3p over-expression in standard culture conditions induced an increase of *Pou3f4* (+77%), supporting the hypothesis that miR-132-3p might be involved in cell identity (Figure 5A). Inhibition of miR-132-3p had no impact on the regulation of this gene (Figure 5B). Expression of the cellular proliferation-related markers, *Mki67* (+60%) and *Tpx2* (+34%) genes, was also increased by miR-132-3p overexpression (Figure 5C) and decreased by miR-132-3p inhibition by 28% for *Mki67* and 24% for *Tpx2* (Figure 5D). Then we measured the effects of miR-

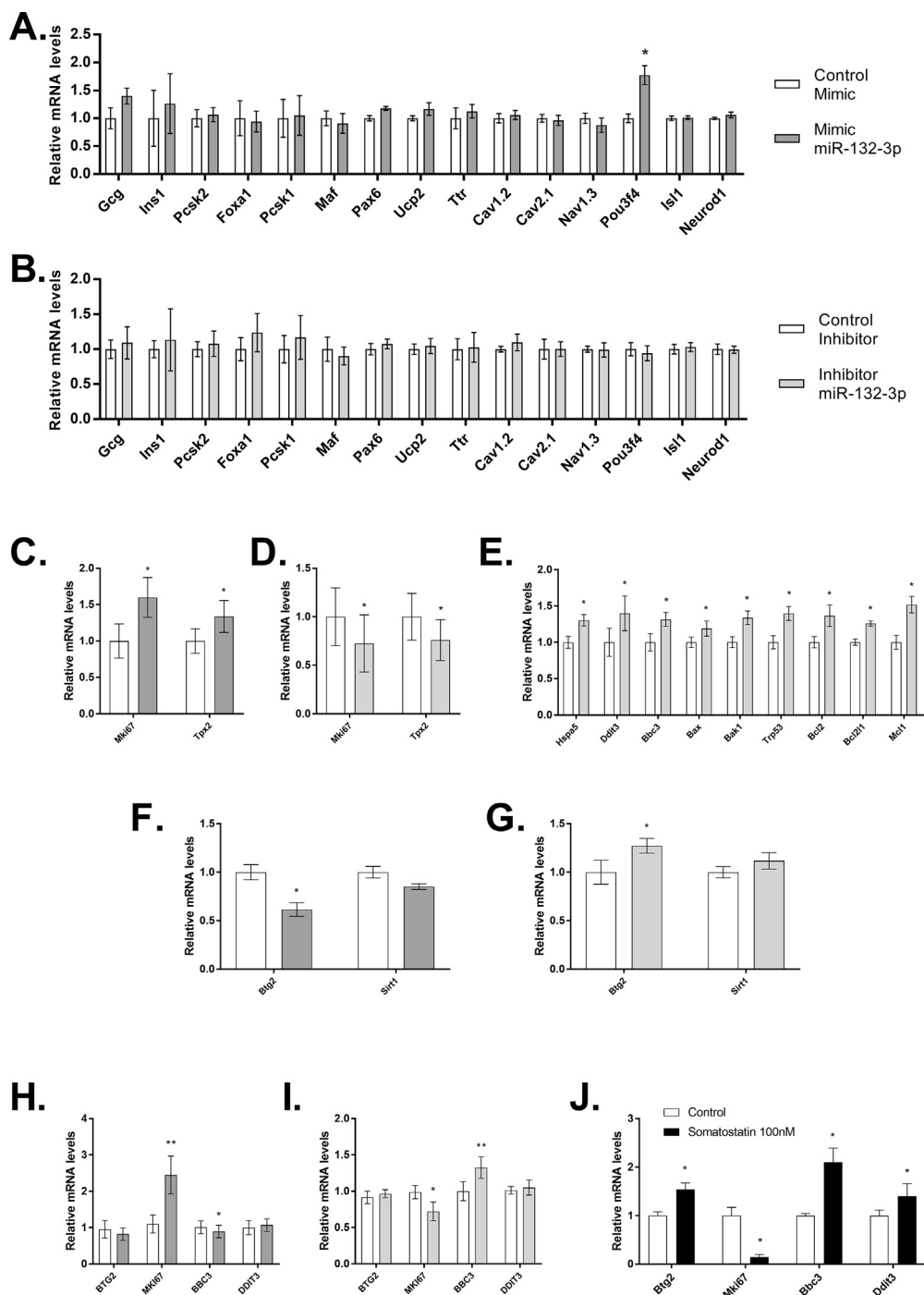


Figure 5: Impact of miR-132-3p on alpha-cell gene expression. (A,B) Evaluation by qPCR of the expression of genes coding for proteins implicated in glucagon synthesis, secretion and alpha-cell identity in primary mouse alpha-cells transfected with 100 nM control mimic or inhibitor (white bars) or miR-132-3p mimic (dark grey) (A) or inhibitor (light grey) (B) and cultured for 48 h in 10% FBS supplemented DMEM with 5.6 mM glucose (n = 4–9). Multiple t-test with Benjamini, Krieger and Yekutieli method, * FDR \leq 0.05 vs control. (C,D) Expression of genes coding for cell proliferation markers measured by qPCR in primary mouse alpha-cells transfected with 100 nM control mimic or inhibitor (white bars) or miR-132-3p mimic (dark grey) (C) or inhibitor (light grey) (D) and cultured for 48 h in 10% FBS supplemented DMEM with 5.6 mM glucose. Two-tailed paired Student's t test, *P \leq 0.05 vs control. (E) Expression of genes coding for cell stress markers measured by qPCR in primary mouse alpha-cells transfected with 100 nM control inhibitor (white bars) or miR-132-3p inhibitor (light grey) and cultured in 1% FBS supplemented DMEM with 0.5% BSA and 5.6 mM glucose. Two-tailed paired Student's t test, *P \leq 0.05 vs control. (F,G) Potential gene targets of miR-132-3p measured by qPCR in primary mouse alpha-cells transfected with 100 nM control mimic or inhibitor (white bars) or miR-132-3p mimic (dark grey) (F) or inhibitor (light grey) (G) and cultured for 48 h in 10% FBS supplemented DMEM with 5.6 mM glucose. Two-tailed paired Student's t test, *P \leq 0.05 vs control. (H–I) Btg2 and Mki67 mRNA levels measured by qPCR in human primary alpha-cells transfected with 100 nM control mimic or inhibitor (white bars) or miR-132-3p mimic (dark grey) (H) or inhibitor (light grey) (I) and cultured in PIM-S medium supplemented with 10% human serum for 48 h. One-tailed paired Student's t test, *P \leq 0.05 vs control. (J) Btg2, Mki67 Bbc3 and Ddit3 mRNA levels measured by qPCR in primary mouse alpha-cells in control condition or treated for 48 h with 100 nM somatostatin in 1% FBS supplemented DMEM with 0.5% BSA and 5.6 mM glucose. One-tailed paired Student's t test, *P \leq 0.05 vs control.

132-3p inhibition on the expression of genes related to cellular stress and apoptosis in control (0.5% BSA vehicle) and glucolipotoxicity conditions (0.5% BSA + 0.5 mM palmitate + 25 mM glucose). miR-132-3p inhibition induced an increase of the endoplasmic reticulum stress markers *Hspa5*, *Ddit3* as well as of the pro-apoptotic genes *Bbc3*, *Bax*, *Bak1* and *Trp53*. Moreover, miR-132-3p inhibition also increased anti-apoptotic *Bcl2*, *Bcl2l1* and *Mcl1* genes expression (Figure 5E). Of interest, in glucolipotoxicity conditions, we also observed an increase of *Hspa5*, *Bbc3*, *Bak1*, *Trp53*, *Bcl2* and *Bcl2l1* genes expression after miR-132-3p inhibition (Supp Fig. F). In order to identify possible direct target mRNAs of miR-132-3p implicated in proliferation and apoptosis resistance, we evaluated the expression of the *Btg2* and *Sirt1* genes, two computationally predicted and validated targets of miR-132-3p with anti-proliferative and stress resistance associated functions. We observed a 39% decrease and a 27% increase of *Btg2* gene expression in primary mouse alpha-cells treated with miR-132-3p mimic and inhibitor, respectively. In contrast, *Sirt1* was not significantly affected by miR-132-3p (Figure 5F,G). This supports the hypothesis that *Btg2* is a possible miR-132-3p direct target and that miR-132-3p has multiple gene targets involved in cell proliferation and death as well as cellular stress markers. To confirm the possible implication of miR-132-3p in the regulation of human alpha-cells proliferation and apoptosis, we evaluated the molecular impact of miR-132-3p overexpression and downregulation in human primary alpha cells. Like in mouse alpha-cells, the overexpression of miR-132-3p induced an increase of the proliferation marker MKI67 (+121%) and a decrease of BBC3 mRNA level (−12%) without changes of DDIT3 gene expression, while the inhibition of miR-132-3p induced a decrease of MKI67 (−28%) expression and increase of BBC3 gene expression (+32%) without change of DDIT3 mRNA level. BTG2 expression was not affected in human alpha cells by the modulation of miR-132-3p, suggesting that it is not a direct target in these cells, at least at the mRNA level (Figure 5H,I).

3.7. Molecular impact of somatostatin treatment

As somatostatin is a negative modulator of miR-132-3p expression *in vitro*, we hypothesized that this hormone could regulate alpha-cell proliferation and apoptosis via miR-132-3p. The treatment of mouse primary alpha-cells for 48 h with 100 nM somatostatin induced an increase of *Btg2* (+54%), a decrease of the proliferation marker *Mki67* (−86%) and an increase of pro-apoptotic genes *Bbc3* (+110%) and *Ddit3* (+40%), reproducing the principal effects of miR-132-3p inhibition although at higher magnitude (Figure 5J).

4. DISCUSSION

We identified changes of miRNA expression between obese hyperglycemic and control mice, 16 miRNAs in alpha-cells, and 28 miRNAs in beta-cells. Previous microarray analyses were performed by Nesca et al. on whole islets from HFD and db/db mice compared to their controls. In this study, 79 miRNAs were identified with a regulation level higher than 1.5 fold in HFD versus normal diet fed mice [29]. Of the 16 miRNAs we identified as regulated in alpha-cells, only one was identified by Nesca et al., indicating that alpha-cells have to be purified to be studied at the molecular level. We observed a lower regulation level of miRNAs compared to the study by Nesca et al. in whole islets. Although we have no clear explanation for these differences, we hypothesize that it could be due to the longer isolation process, adding trypsin digestion and FACS sorting steps, or to the low and high fat feeding protocols substantially different in their composition and duration.

Alpha- and beta-cells miRNA expression profiles in LFD mice showed a signature of 125 miRNAs significantly different between both cell types. Among these 125 miRNAs, 63 were more expressed in alpha-cells and 62 more in beta-cells. miR-205-5p and miR-92a-3p were the most alpha-cell specific. This balanced numbers of alpha- and beta-cell enriched miRNAs differs with the results obtained with human alpha- and beta-cells sorted after dissociation of prefixed islets. In the study of Klein et al., 95% of the differentially expressed miRNAs were more abundant in beta-cells [33]. The comparison of alpha- and beta-cells miRNAs was also conducted by comparing β TC3, a beta-cell line, to α TC1-6, a mouse adenoma pancreatic alpha-cell line, in two different studies, leading to the identification of 9 and 50 miRNAs preferentially expressed in beta-cells while 14 and 74 were preferentially expressed in alpha-cells. α TC1-6 enriched miRNAs included miR-103, miR-130 and miR-146a which were also alpha-cells enriched miRNAs in our study [34,35].

Among the 16 miRNAs regulated in alpha-cells of obese hyperglycemic mice compared to controls, 8 were located in the *Meg3-Mirg* cluster: miR-487b-3p; miR-369-5p; miR-382-5p; miR-409-3p; miR-431-5p; miR-127-3p; miR-329-3p; miR-382-3p. This genomic imprinted region was described as controlled by epigenetic regulation of two differentially methylated regions (DMRs) in different models. The intergenic germline-derived differentially methylated region (IG-DMR) located in chromosome 12, ~12 kb upstream of the MEG3 promoter, is subjected to allele-specific imprinting and was recognized as a major distal regulatory element controlling the expression of the 200 kb downstream microRNA containing genes, including the MEG3-MIRG cluster [36]. In human type 2 diabetic islets, hypermethylation of the MEG3-DMR, located in MEG3 promoter, 1.5 kb upstream of the transcription initiation, is correlated with a downregulation of miRNAs from the cluster [19]. The average regulation of these 8 miRNAs is a 1.33 fold decrease. The microarray technology used cannot indicate if this regulation is due to a weak regulation in each cell or to a strong regulation in part of the cells. Indeed, the existence of alpha-cell subpopulations was already described using single-cell transcriptomic approaches [37]. In induced pluripotent stem cells [iPSCs], low levels of DNA methylation in the IG-DMR enhancer is necessary to maintain the expression of lncRNAs and miRNAs essential for the establishment of full pluripotency [38]. Functionally, downregulation of the cluster in alpha-cells could be a modulator of possible transdifferentiation or dedifferentiation processes in part of alpha-cells.

The *in vitro* molecular and functional studies were focussed on miR-132-3p as it represents the most regulated miRNA between obese hyperglycemic and control mice in alpha-cells. miR-132-3p was the only miRNA with a regulation-fold higher than two. However neither palmitate, high glucose concentration alone or in combination, nor insulin led to any change in miR-132-3p expression. It is possible that 48 h treatments are not long enough to reproduce the changes occurring during the 16 weeks of HFD feeding *in vivo* or that glucolipotoxicity *in vivo* includes much more than high glucose, fatty acids and amino acids. Nevertheless, in isolated rat whole islets, miR-132 is regulated by 48 h palmitate treatment in the presence of high glucose concentrations, suggesting a different regulatory mechanism in beta-cells [29]. Interestingly, miR-132-3p that we found downregulated in sorted alpha-cells of HFD mice at 16 weeks, shows an opposite regulation in sorted beta-cells as previously reported in islets of obese and db/db mice as well as in the non-obese type 2 diabetes Goto-Kakizaki rats [29,39]. The difference between alpha- and beta-cells could be due to transcriptional positive

regulations of the miR-212/miR-132 cluster by GLP-1 through a cAMP-dependent pathway in beta-cells [20,28] while alpha-cells, expressing very low levels of GLP-1 receptor, would be not or weakly receptive [40]. On the other hand, we can postulate that somatostatin produced within the islet by delta-cells may mediate the negative regulation of miR-132-3p in alpha-cells. Indeed, somatostatin, which can be hypersecreted in obese rats [41] and emerges as a critical modulator of alpha-cell function in controlling glucagon secretion, and alpha-cell mass [42–44], reduced strongly miR-132-3p expression *in vitro* in our study. We hypothesize, that GLP-1 by stimulating somatostatin secretion in the pancreas may reduce cAMP through the $G_{i/o}$ coupled receptor Sst2 and repress miR-132-3p expression in the same way that it inhibits glucagon secretion [42,45]. The increased plasma GLP-1 in our model of obese hyperglycemic mice [8] could so be a central regulator of miR-132-3p in both alpha- and beta-cells.

Functionally, neither miR-132-3p inhibition, nor its overexpression, had an impact on basal as well as arginine- or low glucose-stimulated glucagon secretion in opposition to its effects on glucose-stimulated insulin secretion in beta-cells [20,28,29]. However, like in beta-cells, miR-132 is implicated in alpha-cells proliferation [29]. miR-132-3p overexpression increased primary mouse alpha-cell proliferation and its inhibition decreased the proliferation rate. This is corroborated by molecular analyses in the presence of miR-132-3p mimics or inhibitors on the proliferation markers, *Mki67* and *Tpx2*, which were increased by over-expression of miR-132-3p and decreased by its inhibition in correlation with proliferation in mouse alpha-cells. We showed that the BTG anti-proliferation factor 2 (*Btg2*) mRNA, a previously reported direct target of miR-132-3p, is also regulated in mouse alpha-cells and potentially implicated in this process by its regulation in an opposite way to miR-132-3p [46]. Interestingly, *Btg2* mRNA expression is inversely correlated to the expression of miR-132-3p between mouse alpha- and beta-cells (unpublished data). We also observed increased expression of the transcription factor *Pou3f4* gene, implicated in alpha-cell identity, after miR-132-3p over-expression while miR-132-3p inhibition was without effects [47,48]. High expression level of miR-132-3p might be a positive regulator of alpha-cells differentiation, but the results are at odds with the notion that increased cell proliferation is usually accompanied with cell dedifferentiation, leaving this observation to be more fully characterized.

Finally, our data indicate that miR-132-3p is also implicated in alpha-cell resistance to apoptosis. The high expression levels of the Bcl2 family proteins balancing the impact of pro-apoptotic factors in alpha-cells was described as a mechanism of their strong resistance to stress induced apoptosis compared to beta-cells [32]. Interestingly, pro- and anti-apoptotic genes, including *Bcl2* and *Bcl2l1*, were upregulated by the inhibition of miR-132-3p in both standard and glucolipotoxicity conditions. The surprising finding of the upregulated pro- and anti-apoptotic gene expression, induced by miR-132-3p downregulation even in normal culture conditions leads to hypothesize that, in low miR-132-3p levels, the ratio of pro- and anti-apoptotic factors is changed in favor of pro-apoptotic factors, leading to the functional activation of proteins directly involved in apoptosis induction such as BAX and BAK [49].

The impact of miR-132-3p modulation was partially identical in primary culture of human alpha-cells where miR-132-3p appears as a positive regulator of the proliferation marker gene MKI67 and a repressor of the stress marker gene BBC3. Interestingly BTG2 gene expression was not affected in human alpha-cells in contrast to what we observed in mouse alpha-cells. This may indicate that the functions or miR-132-3p are highly conserved through the evolution and may be

mediated by different mRNA targets as reported in a recent bioinformatics study [50].

5. CONCLUSION

In conclusion, the profile of regulated miRNAs in alpha-cells of obese hyperglycemic mice revealed downregulation of miRNAs belonging to the Mirg-Meg3 cluster whose functional impacts on alpha-cell remains to be investigated. Our study, focusing on the most regulated miRNA, miR-132-3p, highlights its potential impact on alpha-cell mass through a mechanism that may involve the regulation of *Btg2*. The 2-fold downregulation of this miRNA, may be a mechanism of action of somatostatin within the islet, restraining proliferation and sensitizing alpha-cells to apoptosis, contributing to the balanced alpha-cell mass of HFD mice. Indeed, even if alpha-cells proliferate during HFD [51], alpha-cells number remains unchanged after 16 weeks of HFD feeding while a strong increase of beta-cell number is observed [8]. miR-132-3p, may be an interesting target in the study of type 2 diabetes pathophysiology by its dual action in alpha- and beta-cell proliferation, apoptosis and resistance to stress during its evolution. At 16 weeks, its decreased expression in alpha-cells could contribute to the adaptation of a proliferative phase of glucagon-producing cells while its upregulation in beta-cells should promote their expansion and insulin secretion.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molmet.2019.01.004>.

CONFLICT OF INTEREST

All authors have no conflicts of interest.

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