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► To cite this version:

Olivier Dumortier, Gaia Fabris, Didier Pisani, Virginie Casamento, Nadine Gautier, et al.. microRNA-375 regulates glucose metabolism-related signaling for insulin secretion. *Journal of Endocrinology*, 2020, 244 (1), pp.189-200. 10.1530/JOE-19-0180 . hal-02400345

HAL Id: hal-02400345

<https://hal.science/hal-02400345>

Submitted on 26 Nov 2020

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microRNA-375 regulates glucose metabolism-related signaling for insulin secretion

Olivier Dumortier^a, Gaia Fabris^{a,b}, Didier F Pisani^b, Virginie Casamento^a, Nadine Gautier^a, Charlotte Hinault^c, Patricia Lebrun^a, Christophe Duranton^b, Michel Tauc^b, Stephane Dalle^d, Julie Kerr-Conte^e, François Pattou^e, Marc Prentki^f and Emmanuel Van Obberghen^{b,c,g*}

^a Université Côte d’Azur, Inserm, CNRS, IRCAN, France

^b Université Côte d’Azur, CNRS, LP2M, France

^c Université Côte d’Azur, CHU, Inserm, CNRS, IRCAN, France

^d INSERM U1191, Institute of Functional Genomics (IGF), CNRS UMR5203, Montpellier University, Montpellier, France

^e Translational Research for Diabetes, University of Lille, INSERM, CHRU Lille, France.

^f CRCHUM and Montreal Diabetes Research Center and Departments of Nutrition and Biochemistry and Molecular Medicine, University of Montreal, Canada

^g Université Côte d’Azur, CHU, CNRS, LP2M, France

* Corresponding author: Emmanuel Van Obberghen, LP2M CNRS-UMR7370, Medical Faculty, 28 avenue Valombrose, 06107 Nice cedex 2. Mail : emmanuel.van-obberghen@unice.fr

Short title. microRNA-375 modulates insulin secretion

Keywords. diabetes, glucose metabolism, insulin secretion, microRNAs, pancreatic islets.

Word count. 4621 (from title to end of discussion)

Abstract

Enhanced beta cell glycolytic and oxidative metabolism are necessary for glucose-induced insulin secretion. While several microRNAs modulate beta cell homeostasis, microRNA-375 stands out as it is highly expressed in beta cells where it regulates beta cell function, proliferation and differentiation. As glucose metabolism is central in all aspects of beta cell functioning, we investigated the role of microRNA-375 in this process using human and rat islets; the latter being an appropriate model for in depth investigation. We used forced expression and repression of microRNA-375 in rat and human primary islet cells followed by analysis of insulin secretion and metabolism. Additionally, microRNA-375 expression and glucose-induced insulin secretion were compared in islets from rats at different developmental ages. We found that overexpressing of microRNA-375 in rat and human islet cells blunted insulin secretion in response to glucose but not to α -ketoisocaproate or KCl. Further, microRNA-375 reduced O₂ consumption related to glycolysis and pyruvate metabolism, but not in response to α -ketoisocaproate. Concomitantly, lactate production was augmented suggesting that glucose-derived pyruvate is shifted away from mitochondria. Forced microRNA-375 expression in rat or human islets increased mRNA levels of pyruvate dehydrogenase kinase-4, but decreased those of pyruvate carboxylase and malate dehydrogenase1. Finally, reduced microRNA-375 expression was associated with maturation of fetal rat beta cells and acquisition of glucose-induced insulin secretion function. Altogether our findings identify microRNA-375 as an efficacious regulator of beta cell glucose metabolism and of insulin secretion, and could be determinant to functional beta cell developmental maturation.

46 **Abbreviations**

47 GSIS, Glucose-stimulated insulin secretion ; KIC, alpha-ketoisocaproate ; KRBH, Krebs-
48 Ringer Bicarbonate-Hepes buffer ; Ldh, lactate dehydrogenase ; LP diet, low protein diet ;
49 Mdh1, malate dehydrogenase 1 ; Mpc, mitochondrial pyruvate carrier ; miR, microRNA ; miR-
50 375, microRNA-375 ; Pc, pyruvate carboxylase ; Pdh, pyruvate dehydrogenase ; Pdk, pyruvate
51 dehydrogenase kinase ; TCA cycle, tricarboxylic acid cycle.

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Introduction

The main beta cell mission consists in its ability to secrete insulin for maintenance of organismal glucose homeostasis. This hallmark requires expression of a set of genes (allowed genes) and the concomitant repression of others (disallowed genes) (Pullen and Rutter 2013; Quintens, et al. 2008). Indeed, beta cells have a dedicated metabolism comprised of a tightly regulated chain of elements from glucose sensors to a specialized exocytosis machinery. Glucose metabolism in beta cells linked to insulin secretion has several exclusive features. First, glucose transport is not limiting for its metabolism, and it results in a rapid equilibrium between intracellular and extracellular glucose at all glycemic levels. The second particular trait is that glycolysis is initiated by glucokinase having a high K_m for glucose associated to an elevated V_{max} , and being devoid of feedback control (Prentki, et al. 2013). As beta cells express low levels of plasma membrane monocarboxylate transporters, little or no monocarboxylates, including lactate, enter the cells. Further, the low lactate dehydrogenase (LDH) activity of the beta cells accounts for the fact that a major fraction of the glucose entering the beta cells is oxidized by the mitochondria (Delghingaro-Augusto, et al. 2009; Schuit, et al. 1997). These distinctive beta cell properties must be maintained to achieve efficient oxidative phosphorylation, lipid metabolism and signaling (Prentki et al. 2013), thus making optimal glucose sensing possible. It is thought that beta cell dysfunction in type 2 diabetes could result from a reduced expression of key genes, triggering the loss of beta cell identity and hence promoting dedifferentiation (Delghingaro-Augusto et al. 2009; Talchai, et al. 2012; Weir, et al. 2001). Importantly, grounds have been gleaned in favor of beta cell dedifferentiation in human type 2 diabetes. Indeed, dedifferentiated cells account for almost 40% of the beta cells in type 2 diabetes patients compared to 8% in controls (Cinti, et al. 2016). Thus, it appears that the maintenance of beta cell identity throughout life is crucial for glucose homeostasis and organismal physiology.

The biological importance of microRNAs is substantiated by the diverse and profound phenotypic consequences occurring upon changes in their expression. These modifications are associated with physiological modulations, but also with perturbed development and pathological situations. Thus, microRNAs have emerged as major modulators of gene expression in many biological programs including organ development and metabolism (Dumortier, et al. 2013).

One of the most relevant and widely explored microRNAs in beta cells is miR-375 (Dumortier, et al. 2016; Guay and Regazzi 2015; Martinez-Sanchez, et al. 2017), which is preferentially expressed in islets and is the most abundant microRNA in adult beta cells (Poy, et al. 2004).

We and others demonstrated that this microRNA is a chief regulator of beta cell mass and functions through incompletely understood mechanisms (Dumortier, et al. 2014; El Ouaamari, et al. 2008; Latreille, et al. 2015; Poy, et al. 2009). In addition, several studies have found that miR-375 expression is upregulated in islets from type 2 diabetics (Bloomston, et al. 2007; He, et al. 2017; Zhao, et al. 2010). Similarly, we observed in a murine model of predisposition to type 2 diabetes following a low protein diet (LP) during pregnancy, that miR-375 expression is increased in the endocrine pancreas of the progeny (Dumortier et al. 2014). This is accompanied by a reduction in both glucose-stimulated insulin secretion (GSIS) and beta cell proliferation.

Anti-miR-induced normalization of miR-375 expression levels in the islets of LP descendants partially restored insulin secretion and cell proliferation to levels of control animals. However, it should be noted that no consensus exists on the differential expression of miR-375 in the healthy versus the type 2 diabetic condition as reported by other studies in humans (Ofori, et al. 2017; Seyhan, et al. 2016) or animals (Esguerra, et al. 2011), in which no increased miR-375 expression was observed in the disease situation. This missing general agreement could be due, at least in part, to the pronounced heterogeneity of type 2 diabetes concerning the underlying

105 pathophysiology, epigenetic/genetic landscape, disease progression and degree of metabolic
106 perturbations.

107 While it is generally accepted that miR-375 is essential for the development of the endocrine
108 pancreas (Avnit-Sagi, et al. 2009; Joglekar, et al. 2009; Kloosterman, et al. 2007), facts in favor
109 of additional roles are appearing. Indeed, evidence is being built highlighting an association
110 between low miR-375 levels and beta cell dedifferentiation (Nathan, et al. 2015). Further, it
111 has been recently found *in vitro* that miR-375 overexpression is sufficient to generate insulin-
112 producing cells from pluripotent stem cells or from human mesenchymal stem cells (Shaer, et
113 al. 2014a; Shaer, et al. 2014b). Taken together the striking features of miR-375 action in beta
114 cells, *i.e.* inhibition of GSIS and promotion of beta cell differentiation, reveal at first glance a
115 potentially discordant image.

116 Beta cell differentiation is a complex multistep process finely orchestrated by numerous
117 transcription factors. The ultimate beta cell maturation step is thought to occur around weaning.
118 It consists in the acquisition of glucose stimulus-secretion coupling which depends on the
119 occurrence of glucose metabolism dedicated to insulin secretion (Stolovich-Rain, et al. 2015).
120 As glucose metabolism governs insulin secretion and is inherent to mature beta cells we posit
121 here that the miR-375 effects on beta cell function could be due to glucose metabolism
122 alterations.

123

Materials and methods

Reagents

Culture media and buffer solutions were from Gibco (ThermoFisher Scientific, Gometz le Châtel, France), FBS and trypsin from Invitrogen (Cergy Pontoise, France). Other reagents were from Sigma-Aldrich Chimie (Saint-Quentin Fallavier, France).

Human islets

Islets were provided by the Islets for Research distribution program through the European Consortium for Islet Transplantation under the supervision of the Juvenile Diabetes Research Foundation (31-2012-783). Human pancreases were harvested from 5 adult brain-dead individuals (males=2, females=3; age, mean \pm SEM: 44.0 \pm 10.0 years; BMI, 21.0 \pm 1.0 kg/m²; HbA1c, 36 \pm 1 mmol/mol (5.4 \pm 0.1%)) in agreement with French regulations and Lille University Ethical Committee. Experiments used islets with >90% viability and >80% purity (endocrine versus exocrine tissue).

Animals and diets

All procedures followed ARRIVE guidelines, were conducted in accordance with EU directives for animal experiments (2010/63/EU) and were approved by the French Research Ministry (MESR 00500.02). Three month-old Wistar rats (Janvier; Le Genest Saint Isle, France) were kept under conventional conditions, with free access to water and food. Nulliparous female rats (200–250 g) were mated with male rats and used at day 21 of gestation.

Islet isolation

3 month-old rat islets were obtained after density gradient centrifugation in histopaque-1077 (Sigma) as previously described (Theys, et al. 2011). Hand-picked islets were cultured in

149 RPMI-1640 medium supplemented with FBS (10%, v/v) and antibiotics. Neoformed fetal rat
150 islets were obtained as described (Dumortier, et al. 2011).

152 *Culture and transfection of dissociated islet cells*

153 Rat or human islets were dissociated by trypsinization, and seeded at a density of 25×10^3
154 cells/cm² in dishes coated with 804G-ECM (Dumortier et al. 2014; Parnaud, et al. 2009). 48 h
155 after plating, cells were transfected using Lipofectamine 2000 (Invitrogen) and with double-
156 stranded RNA oligonucleotides (miRIDIAN microRNA mimic, Dharmacon) corresponding to
157 mature human and rattus miR-375-3p (ref. C-320580-01, sequence:
158 UUUGUUCGUUCGGCUCGCGUGA) or control microRNA (ref. CN-001000-01, sequence:
159 UCACAACCUCCUAGAAAGAGUAGA) at a concentration of 100 nmol/l. Single-stranded
160 miRCURY LNA inhibitor (Exiqon, QIAGEN, Courtaboeuf) specifically blocking endogenous
161 miR-375-3p (ref. YI04101398), and its control (ref. YI00199006) were used at a concentration
162 of 100 nmol/l. All analyses were performed 72 h after transfection.

164 *Cytosolic Ca²⁺ determination*

165 72 h post transfection the dissociated islet cells were preincubated for 45 min at 37°C in 1 mL
166 KRBH containing 3 mM glucose, 0.1% BSA (w/v) and 2.5 mM probenidol (Sigma). The
167 dissociated cells were then loaded with Fura-2-AM (2.5 mM; Molecular Probes, Göttingen,
168 Germany) for 30 min. Fluorescence was measured with an inverted phase-contrast microscope
169 (Axiovert 100, Zeiss, Oberkochen, Germany). Cells were excited at 340 and 380 nm, and the
170 fluorescence intensity was recorded at 505 nm. Data were acquired using Axon Imaging
171 Workbench (Axon Instruments, Foster City, CA, USA).

173 *RNA extraction and RT-qPCR*

174 RNAs were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's
175 instructions and RNA quality was tested by UV spectrophotometry on a NanoDrop 1000 device
176 (Thermo Fisher Scientific).

177 For miRNA analysis, 10 ng of total RNA was reverse transcribed using Universal cDNA
178 synthesis kit (Exiqon). qPCR reactions were prepared using SYBR Green master mix, Universal
179 RT (Exiqon) and microRNA LNA PCR primers (Exiqon) according to the manufacturer's
180 manual. qPCR and data evaluation were performed using StepOnePlus apparatus and associated
181 software (Applied Biosystem, ThermoFisher). Data were analyzed using $\Delta\Delta C_t$ method with U6
182 and 5S (control primers set from Exiqon ref. YP00203906 and YP00203907) as endogenous
183 controls and "miR-CTL group" as control condition.

184 For mRNA analysis, 1 μ g of RNA was reverse-transcribed into cDNA using M-MLV-RT and
185 analyzed using SYBR Green (Applied Biosystem, ThermoFisher) and a StepOnePlus apparatus
186 and associated software (Applied Biosystem, ThermoFisher). Data were analyzed using $\Delta\Delta C_t$
187 method with Cyclophilin A as housekeeping and "miR-CTL group" as control condition.

188 The primers sequences are displayed in Table 1.

190 *Lactate determination*

191 Lactate concentration was determined by ionic chromatography using a Dionex DX600 device
192 equipped with an ion Pac AS11 column (ThermoFisher). Sequential anion elution including
193 lactate was obtained at 1 ml/min by a KOH gradient generated by the EG40 eluent generator
194 according to the manufacturer's instructions.

196 *Insulin secretion*

197 All experiments were performed using Krebs-Ringer Bicarbonate-Hepes buffer (KRBH)
198 containing 3 mmol/l glucose and 0.1% BSA (w/v). Dissociated islet cells were pre-incubated

for 1h at 37 °C before incubation in fresh medium containing secretagogues. After 1h, media and cell homogenates obtained by sonification were analyzed for insulin content using a rat insulin ELISA kit (Mercodia AB, Uppsala, Sweden). To eliminate variations due to differences in cell number, insulin secretion was expressed as the percentage of the cellular insulin content, which is referred to as fractional insulin release.

Glucose uptake

Islet cells were preincubated for 45 min at 37°C in 3 mmol/l glucose KRBH and then 10 min in KRBH containing 20 mmol/l glucose and 0.5 uCi of [³H] deoxyglucose (Amersham Pharmacia Biotech, Uppsala, Sweden). Glucose incorporation was stopped on ice and cells were lysed using NaOH 0.2N and neutralized with HCl 0.2N. Radioactivity was measured and the total protein amount recorded using BCA method. Results are expressed in cpm/mg protein/min.

Mitochondrial metabolism

For metabolic analysis, dissociated cells were seeded in a 24 multi-well plate (Seahorse, Agilent, Santa Clara, CA, USA) and cultured/transfected as described above. The oxygen consumption rate (OCR) was determined using an XF24 Extracellular Flux Seahorse Analyzer (Agilent). Rotenone and antimycin-A (2 µmol/l each) were used to inhibit mitochondrial respiration and oligomycin A (1.2 µmol/l) to inhibit ATP synthase.

Statistical analysis

Data are presented as mean ± SEM. Statistical analyses were performed using InStat3 software (Graphpad, San Diego, CA, USA). Paired two-tailed Student's t tests were used to compare differences between samples from paired experiments. Differences between all other datasets

224 were analyzed by one-way ANOVA with Student-Newman–Keuls post-hoc test. p-values
225 <0.05 were considered significant.

226

Results

miR-375 overexpression in islet cells impairs insulin secretion and the Ca²⁺ rise induced by glucose but not by KCl

We investigated the miR-375 impact on beta cell function by comparing dissociated primary adult rat islet cells transfected with miR-375 or control microRNA (CTL). Double-stranded RNA oligonucleotides corresponding to mature miR-375-3p or control microRNA were first tested in a dose-response experiment from 50 to 200 nmol/l and compared to the untransfected condition (Fig. 1a-c). Augmenting the amount of transfected miR-375 resulted in an increased miR-375 level in the cell (Fig. 1a) and in a decreased insulin secretion in response to 20 mmol/l glucose (Fig. 1b), without affecting the insulin cell content (Fig. 1c). Note that under our experimental conditions 100 nM control oligonucleotide are not deleterious for beta cell function and insulin secretion as these are the same compared to the untransfected condition. Therefore, we choose to use transfection with 100 nmol/l of miR-375 which resulted in an approximately 25-fold increase in miR-375 level and a reduced GSIS by approximately 50% at all glucose concentrations (Fig. 2a).

To evaluate whether this could be attributed to altered exocytosis, we measured insulin secretion at low glucose (3 mmol/l) in the presence of depolarizing KCl concentrations. High miR-375 levels did not modify insulin secretion at the KCl concentrations tested (Fig. 2b). Further, the glucose-induced cytosolic Ca²⁺ rise was almost abolished, whereas that caused by KCl was preserved (Fig. 2c). Together the data are compatible with the view that miR-375 interferes with GSIS by altering beta cell metabolism and metabolic signaling for secretion rather than by hampering late steps in regulation of the exocytosis apparatus.

miR-375 impacts glucose-stimulated respiration

To investigate whether the dampened insulinotropic action of glucose could be due to a miR-375-induced inhibition of glucose uptake, we compared glucose transport using ^3H -2-deoxyglucose in adult rat primary islet cells transfected with control microRNA or with miR-375. As shown (Fig. 2d), no difference in 2-deoxyglucose uptake was observed at 20 mmol/l glucose. To decrypt miR-375 effects on intermediate metabolism, O_2 consumption in response to glucose was evaluated. It was profoundly reduced by miR-375 (Fig. 2e-g) to a similar extent as the GSIS inhibition. Moreover, miR-375 transfected cells must synthesize less ATP in response to glucose as they consume less oxygen (Fig. 2f) with an equivalent coupling to ATP synthesis (Fig. 2g).

Reduced endogenous miR-375 levels in adult rat primary islet cells increases glucose metabolism and insulin secretion

To confirm the repressive miR-375 action on insulin secretion, we decreased the endogenous miR-375 level with its antisens. miR-375 reduction in adult rat primary islet cells increased GSIS with no impact on insulin content (Fig. 3a-b). Remarkably, the diminished miR-375 level augmented glucose-induced O_2 consumption (Fig. 3c-d). Together, our gain and loss of function approach identifies miR-375 as a robust insulin secretion modulator which impacts glucose-induced mitochondrial metabolism.

miR-375 does not affect the action of a fuel stimulus enhancing mitochondrial metabolism independently of glycolysis

To further explore miR-375 action on cell metabolism, we used α -ketoisocaproate (KIC), which induces insulin release by directly augmenting the tricarboxylic acid (TCA) cycle intermediate α -ketoglutarate, and also via acetyl-CoA and acetoacetate production. As illustrated in Fig. 4a, KIC stimulated insulin secretion in a dose-dependent manner comparably in control and miR-

375 overexpressing islet cells. Further, mitochondrial metabolism remained unaltered in miR-375-transfected islets in response to KIC, as reflected by O₂ consumption determinations (Fig. 4b-c).

miR-375 modifies pyruvate metabolism

The above results suggest that miR-375 could perturb insulin secretion by interfering with the production of glycolysis metabolites, in particular the last one in the pathway, pyruvate. To examine if miR-375 controls pyruvate mitochondrial metabolism we tested the effect of high pyruvate (50 mmol/l) concentrations on insulin secretion and O₂ consumption. We used an elevated concentration of pyruvate, as it is not an efficacious secretagogue because the plasma membrane monocarboxylate transporter-1 is poorly expressed in adult islets (Pullen, et al. 2012). While pyruvate induced insulin secretion in control islets, its insulinotropic effect in miR-375 transfected islets was diminished (Fig. 4d) to a similar extent as GSIS (Fig. 1a). Forced miR-375 expression also reduced O₂ consumption in response to pyruvate (Fig. 4e). In differentiated beta cells, pyruvate is slightly reduced to lactate and hence most of the glucose-derived pyruvate is metabolized in the mitochondria via pyruvate dehydrogenase and anaplerotic reactions (Ainscow, et al. 2000). However, islet cells overexpressing miR-375 showed a 2-fold increase in lactate release into the medium (Fig. 4f). This suggests that enhanced miR-375 expression partially redirects pyruvate metabolism from mitochondrial oxidation to anaerobic glycolysis and lactate production.

Forced miR-375 expression in human islet cells reduces insulin secretion in response to glucose but not to KCl or to KIC

Next we addressed in human beta cells the role of miR-375 as regulator of glucose metabolism and GSIS. We measured insulin secretion in islets from 5 non-diabetic donors transfected or

not with miR-375. An approximately 12-fold increased miR-375 level 72 h post transfection (Fig. 5a) markedly reduced GSIS, without affecting the insulinotropic action of KIC or KCl (Fig. 5b). Hence, the miR-375 overexpression data on human islet cells reproduce the results obtained with adult rat islet cells in terms of insulin secretion in response to glucose, KIC and KCl.

Forced miR-375 expression in adult rat primary islets and human islet cells causes deregulation of genes involved in specialized beta cell metabolism

To explore the molecular underpinnings of the action of miR-375 on insulin secretion we looked at the gene expression of key actors of mitochondrial metabolism. As shown in Fig. 6a, in rat islets overexpressing miR-375 mitochondrial pyruvate carrier-1 and -2 expression was not affected by miR-375 suggesting that pyruvate's uptake into the mitochondria is unaltered. Once in the mitochondria, pyruvate enters the TCA cycle after its decarboxylation to acetyl CoA by the PDH complex or after its carboxylation to oxaloacetate by pyruvate carboxylase (PC). Interestingly, in miR-375 overexpressing cells, the mRNA level of *Pdk4* (pyruvate dehydrogenase kinase-4) was increased, while that of *Pc* was decreased (Fig. 6a). Together our data support the notion that miR-375 reduces pyruvate oxidation and its conversion via carboxylation into intermediates for the TCA cycle. Further, the mRNA encoding cytosolic malate dehydrogenase1 (*Mdh1*), a component of the malate-aspartate shuttle that transfers reducing equivalents to the mitochondria and the pyruvate-citrate cycle, was downregulated (Fig. 6a). Reduced *Mdh1* transcript levels suggest diminished mitochondrial energy metabolism and cataplerotic signals in response to glucose, that will decrease insulin secretion. Of note, in human islets overexpressing miR-375, similarly to rat islets, *Pc* and *Mdh1* expressions were downregulated, while *Pdk4* mRNA was upregulated (Fig. 6b).

Reduced miR-375 expression is associated with in vitro glucose responsiveness and maturation of fetal rat beta cells

We next explored the possibility that miR-375 could be involved in beta cell metabolic maturation for GSIS and thus interfere with the acquisition of glucose-responsiveness in the transition from fetal to neonatal beta cells. We took advantage of the finding that functional maturation of glucose stimulus-secretion coupling of fetal rat islets can be accelerated *in vitro* by glucose (Sjoholm, et al. 2000a, b). Thus, we measured the expression of miR-375 in untreated fetal islets compared with fetal islets after *in vitro* maturation by glucose (neonatal islets). As illustrated in Fig. 7a, untreated fetal islets did not respond to 20 mmol/l glucose, but they released insulin when stimulated with KIC. However, after *in vitro* maturation, they secreted insulin appropriately in response to high glucose (Fig. 7b). Remarkably, lower glucose responsiveness of insulin release by untreated fetal beta cells was associated with high miR-375 expression compared to neonatal islets (Fig. 7c). Collectively, these results suggest that repression of miR-375 expression participates to the acquisition of glucose stimulus-secretion coupling.

Discussion

Since their discovery, several microRNAs have been implicated in the development and function of beta cells (Dumortier et al. 2016; Guay and Regazzi 2015). Amongst these, miR-375 stands out as it is highly expressed and regulates not only beta cell development and proliferation, but also its function (El Ouaamari et al. 2008; Jafarian, et al. 2015; Kloosterman et al. 2007; Nathan et al. 2015; Poy et al. 2004; Poy et al. 2009). However, while it is accepted that miR-375 is essential for endocrine pancreas development (Avnit-Sagi et al. 2009; Joglekar et al. 2009; Kloosterman et al. 2007), its inhibitory action on insulin secretion reveals its multiple facets in beta cell physiology. Thus, deciphering miR-375 precise functions and mode of action is of considerable interest. To this end, using primary rat and human islets, we delved into the miR-375 effects on glucose metabolism, that governs insulin secretion and modulates beta cell characteristics. Overall our data support the view that miR-375 inhibits GSIS by modifying the expression of a series of genes tightly linked to metabolic signaling for GSIS, including *Pc*, *Pdk4* and *Mdh1*. *In silico* analysis, using microRNA target prediction software, did not propose their mRNAs as potential miR-375 targets. Thus, we can assume that miR-375, by targeting one or several undefined transcripts upstream of these key metabolic enzymes may regulate beta cell function linked to insulin secretion induced by glucose.

Insulin secretion in immature beta cells is characterized by a poor responsiveness to glucose but a mature-like sensitivity to amino acids (Bliss and Sharp 1992; Hellerstrom and Swenne 1991). In addition, immature islets consume more oxygen at low glucose compared with mature islets but their ability to increase oxygen consumption in response to high glucose is considerably lesser compared with mature islets. This clearly suggests that beta cell maturation is associated with profound metabolic changes (Stolovich-Rain et al. 2015). Transcriptomic analyses to determine the basis of poor glucose responsiveness of neonatal beta cells, compared with adult beta cells, revealed that many genes are differentially expressed, including those

encoding enzymes of mitochondrial shuttles, *Pc* and *carnitine palmitoyl-transferase 2* (Jermendy, et al. 2011). In addition, *Ldh* was significantly elevated in neonatal beta cells, which is particularly interesting as efficient *Ldh* expression would divert pyruvate away from the mitochondria towards lactate production. This scenario is compatible with the idea that metabolic specialization of adult beta cells for generating ATP and additional anaplerosis-derived metabolic coupling factors from aerobic glycolysis is deficient in immature neonatal beta cells (Jermendy et al. 2011). However, glucose exposure of immature beta cells fosters their metabolic maturation. Indeed, nutrient shifts at weaning, induced by replacement of fat-enriched maternal milk with a carbohydrate-rich diet, drive postnatal beta cell maturation via islet-specific microRNAs (Jacovetti, et al. 2015). While in the latter report no alterations in miR-375 expression were observed, the entirely different developmental stages examined and experimental conditions used in our study very likely account for the divergence in microRNA expression. It is well known that fetal rat islets cultured with 11 mmol/l glucose acquired near-adult levels of insulin secretion in response to high glucose (Freinkel, et al. 1984). Here we confirmed this and further demonstrated such decreased miR-375 expression in fetal rat islets matured in 11 mmol/l glucose. By contrast, forced miR-375 expression in mature rat and human beta cells resulted in mirror effects on GSIS. Further, we found that upon miR-375 overexpression mature beta cells display enhanced lactate production.

At variance with previous reports using MIN-6 insulinoma cells or primary mice β -cells (Latreille et al. 2015; Poy et al. 2004; Poy et al. 2009), overexpression of miR-375 has no impact on insulin exocytosis in dissociated islet cells from rat or human origin. Indeed, in islets from both species, KCl-induced insulin secretion was preserved upon overexpression of miR-375. The reason for this discrepancy is unclear, but one possible explanation could be different animal species. Previously, when studied in the MIN-6 line, miR-375 has been implicated in limiting insulin exocytosis by downregulating myotrophin (Mtpn) transcripts (Poy et al. 2004).

However, we were unable to reproduce this MTPN decrease in dissociated primary rat islet cells transfected with miR-375 (*not shown*). Others confirmed our findings, showing that MTPN mRNA did not change following miR-375 overexpression in a cell line from rat origin (INS-1 832/13) (Salunkhe, et al. 2015) or in human β -cells (Nathan et al. 2015). In fact, the implication of MTPN in the inhibition of insulin secretion observed with miR-375 has been derived from studies using for the greater part the MIN-6 cell line. As the miRNA profile of this cell-line differs from that seen in primary β -cells (LaPierre and Stoffel 2017), caution should be exerted in extrapolating the MTPN implication to more physiological β -cell systems. Together our findings strongly support the view that robust miR-375 expression in human or rat β -cells favors an immature metabolic phenotype with low glucose-responsiveness rather than impacting on the exocytosis machinery *per se*.

Recently, the concept emerged that the reduction in beta cell mass in type 2 diabetes could reflect a loss/fading of their crucial attributes rather than increased apoptosis (Brereton, et al. 2016; Jeffery and Harries 2016; Talchai et al. 2012). Considering the increased miR-375 expression in islets from animals and humans with type 2 diabetes demonstrated in several studies (Bloomston et al. 2007; He et al. 2017; Poy et al. 2009; Tattikota, et al. 2014; Zhao et al. 2010) , and the current study showing that miR-375 overexpression results in reduced GSIS in human and rat islets, miR-375 appears to be a likely contributor to the fading of essential characteristics of beta cells in type 2 diabetes pathogenesis.

Conclusion

This study enhances our understanding of the diverse roles whereby miR-375 modulates beta cell function. First, we report that miR-375 modulates key beta cell metabolic pathways specifically for GSIS, but not in response to other secretagogues (fuel and non-fuel stimuli). Second, our work, by focusing on metabolism, unearths a novel mode of action of this

microRNA. Third, it provides a plausible mechanism to explain how miR-375 modulates GSIS by redirecting glucose carbons from mitochondrial metabolism to lactate formation, and thus inhibiting glucose-induced ATP generation and the production of additional anaplerosis-derived regulatory metabolites. Finally, our findings uncover relevant links between epigenetic regulators, key beta cell traits and type 2 diabetes pathogenesis. Considering that the beta cell demise is a most challenging conundrum in the diabetes field, understanding the safeguarding of beta cell fundamental properties will be of major importance for leveraging strategies to preserve it in pathophysiological conditions.

Declaration of interests. The authors declare having no conflict of interest.

Funding. This work was supported by INSERM, Université Côte d’Azur, Conseil Régional PACA, Conseil Général des Alpes-Maritimes, Canada Institutes of Health Research, Aviesan/AstraZeneca (Diabetes and the vessel wall injury program), the Agence Nationale de la Recherche [ANR-RPV12004AAA, ANR-11-LABX-0028-01], and the European Foundation for the Study of Diabetes (EFSD/Lilly, European Diabetes Research Program). E.V.O. is affiliated with the FHU OncoAge (<http://www.oncoage.org/>). M.P. holds the Canada Research Chair in Diabetes and Metabolism. Human islet production was supported by the European Genomic Institute for Diabetes (ANR-10-LABX-46) and the European Consortium for Islet Transplantation (Juvenile Diabetes Research Foundation International).

Contribution statement. OD and DFP designed the study, researched data, and contributed to the manuscript. GF, VC, NG, PL, CH, CD, MT, SD, FP and JKC researched and analyzed data. EVO and MP designed the research project, contributed to the manuscript. All authors contributed to the discussion on the manuscript and approve its final version. E.V.O is the guarantor of this work.

Acknowledgements. The authors thank the IRCAN animal housing facility, genomics core and cytometry core (Cytomed).

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Figure legends.

Figure 1. Forced miR-375 expression in dissociated primary adult rat islet cells reduces glucose-induced insulin secretion. 72h after transfection with 50, 100 or 200 nmol/l of double-stranded RNA oligonucleotides corresponding to the mature miR-375 sequence or to a scrambled control miR (CTL), dissociated primary rat islet cells were harvested for RNA extraction or used for insulin secretion experiments. Cells submitted to the same protocol but without oligonucleotides are displayed as control of transfection (NT, not transfected). (a) Measurement of miR-375 content by RT-qPCR. (b-c) Insulin secretion experiments with various glucose concentrations (3 or 20 mmol/l). To eliminate variations due to differences in cell number, insulin secretion (b) is expressed as the percentage of the islet cell insulin content (c), which is referred to as fractional insulin release. Means \pm SEM; n=3. *P < 0.05, miR-375 versus CTL; **P < 0.01, miR-375 versus CTL; ***P < 0.001, miR-375 versus CTL.

Figure 2. Forced miR-375 expression in adult rat primary pancreatic islet cells impairs glucose metabolism and insulin secretion induced by glucose but not by KCl. (a-b) 72 h post-transfection with miR-375, dissociated islet cells were assayed for insulin secretion using various glucose and KCl concentrations. To eliminate variations due to differences in cell number, insulin secretion is expressed as the percentage of the islet cell insulin content, which is referred to as fractional insulin release. (c) 72 h post transfection islet cells were loaded with the fluorescent sensitive Ca^{2+} probe Fura-2 and incubated at low (3 mM) and high (20 mM) glucose or with KCl (30 mM). Next fluorescence was recorded. The Ca^{2+} traces are representative of 3 independent experiments (10 to 20 cells recorded in each experiment). (d) Glucose transport evaluation in dissociated islet cells 72 h post transfection. The results are expressed in cpm/mg protein/min. (e) Mitochondrial O₂ consumption and (f) averages of O₂ consumption determinations made in the absence or presence of 20 mmol/l glucose. (g)

Mitochondrial O₂ consumption coupled to ATP synthase in presence of 20 mmol/L glucose. Means \pm SEM, n = 4 (a-c) or 3 (e-g). *P < 0.05, **P < 0.01, miR-375 versus control (CTL).

Figure 3. Reduced miR-375 expression in adult rat primary pancreatic islet cells increases glucose metabolism and insulin secretion induced by glucose. Glucose-induced insulin secretion (a), insulin content (b) and mitochondrial O₂ consumption (c) were evaluated 72 h after transfection with anti-miR-375 or with anti-CTL. Panel D shows the averages of O₂ consumption. Means \pm SEM, n = 3. **P < 0.01, anti-miR-375 versus anti-CTL.

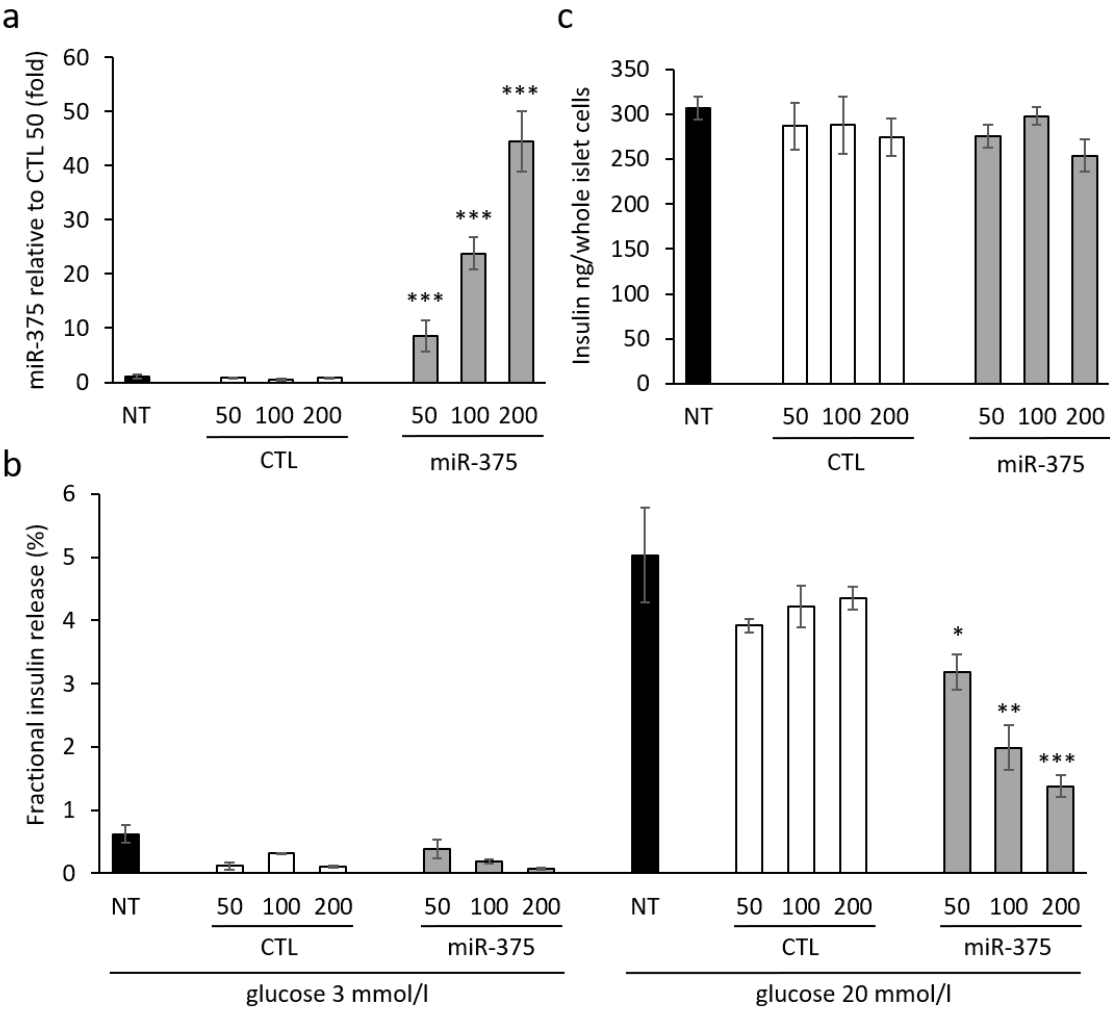
Figure 4. Forced miR-375 expression in adult rat primary pancreatic islet cells impairs pyruvate-induced insulin secretion and pyruvate metabolism with a shift toward lactate production, but does not affect KIC effects. Insulin secretion measured 72 h post-transfection in dissociated islet cells incubated with various concentrations of (a) α -ketoisocaproate (KIC) or (d) pyruvate. O₂ consumption was recorded from cells in presence of KIC (b-c) or pyruvate (e). (f) Lactate measurement in the incubation medium of transfected dissociated islets 4h after glucose addition (11 mmol/l). Means \pm SEM of 3 (a-e) or 4 (f) independent experiments. *P < 0.05, **P < 0.01, miR-375 versus CTL.

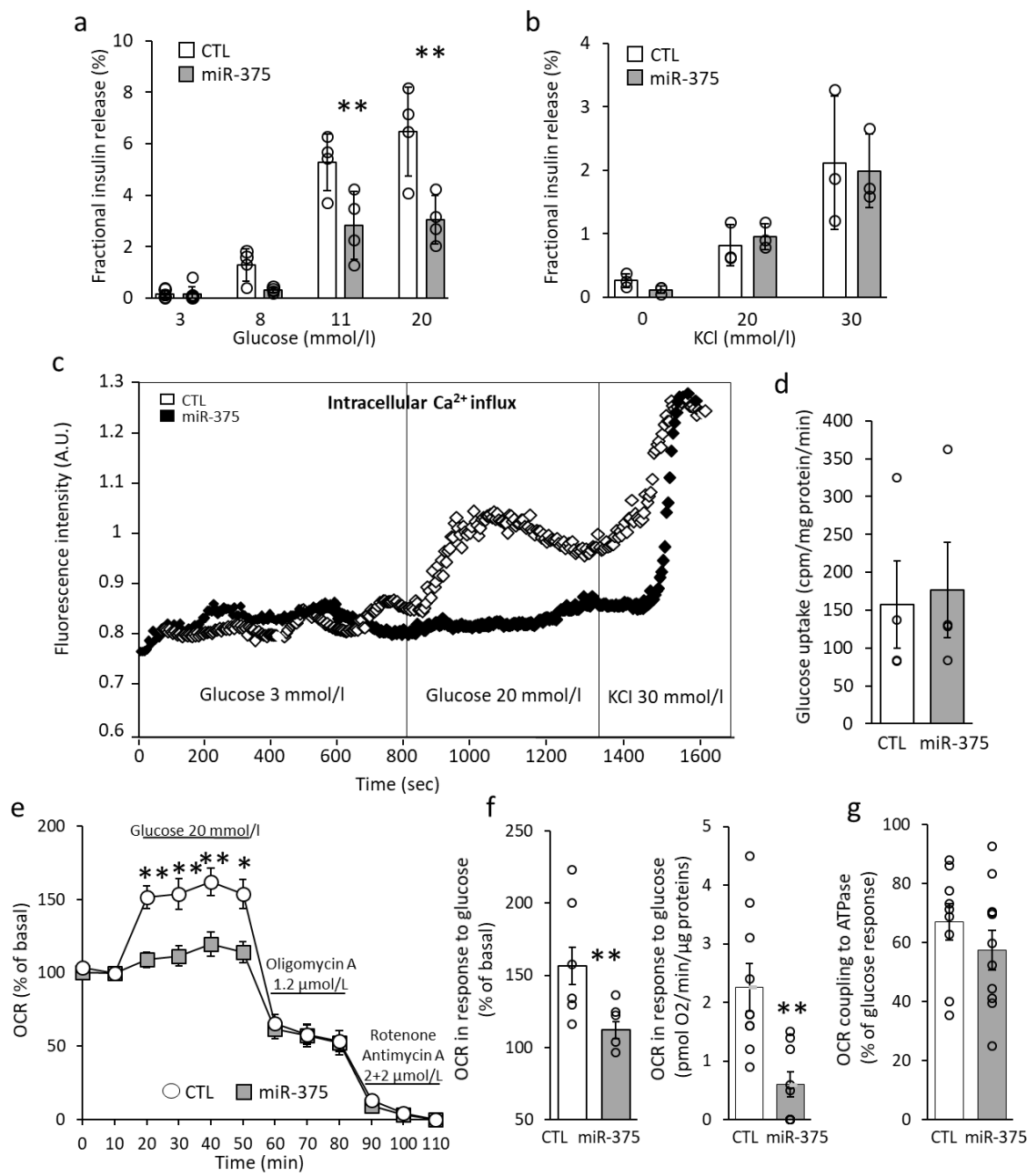
Figure 5. Effect of miR-375 on insulin secretion in human islet cells. (a) miR-375 expression analysed by qPCR in dissociated human islet cells 72 h after miR-375 transfection. (b) Insulin secretion measured in dissociated human islets 72 h after transfection and using various secretagogues. Values are means \pm SEM, n = 5 human islet isolations. *P < 0.05, **P < 0.01, versus CTL.

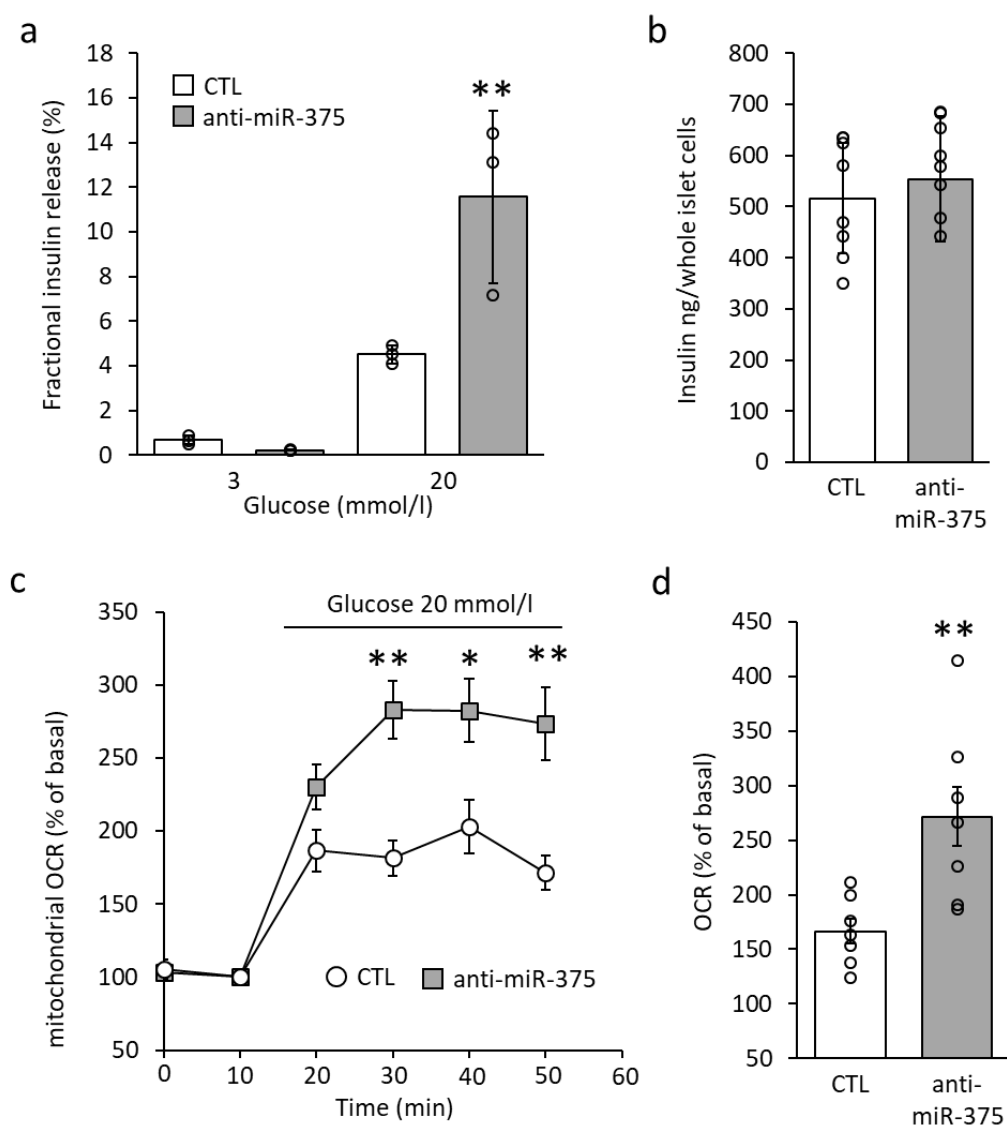
Figure 6. Forced miR-375 expression in adult rat and human islet cells alters the expression level of genes instrumental in β -cell glucose metabolism and metabolic signaling for insulin secretion. mRNA species encoding key metabolic enzymes were measured by qPCR in (a) primary adult rat islets or (b) human islets. Gene expression was

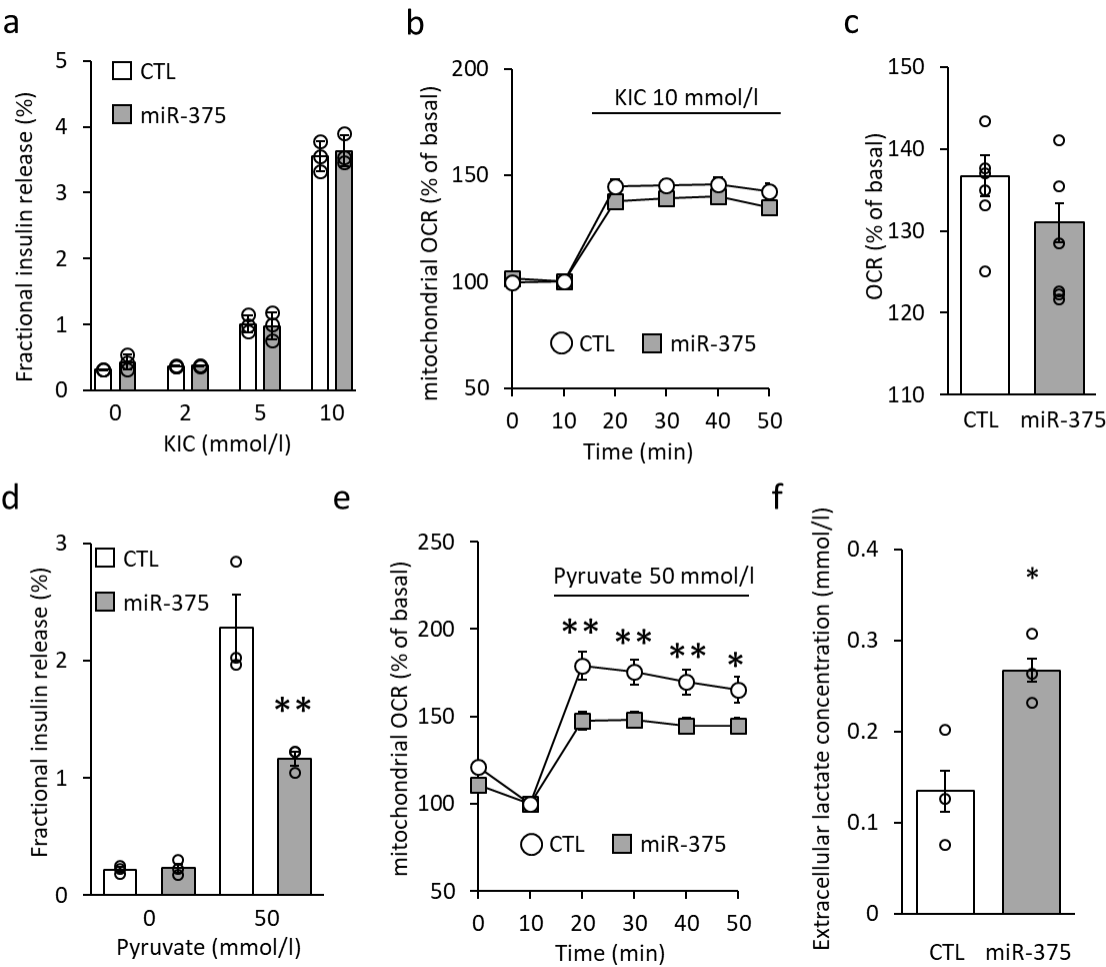
normalized to the cyclophilin A transcript level. Means \pm SEM, n = 6 (a) or 3 (b) independent experiments. Mpc1, Mpc2, mitochondrial pyruvate carrier 1-2; Pdk1-4, pyruvate dehydrogenase kinase 1-4; Pc, pyruvate carboxylase; Mdh1, malate dehydrogenase-1; Ldha, lactate dehydrogenase A. *P < 0.05, miR-375 versus CTL, **P < 0.01, miR-375 versus CTL.

Figure 7. The repression of miR-375 expression is required for the acquisition of the competence for glucose stimulus-secretion coupling. After their digestion, 21 day-old fetal pancreases were either cultured for 7 days in 11 mmol/l glucose for the obtention of “neonatal” islets or purified with density-gradient for fetal islet isolation. (a) Insulin secretion analysis of fetal islets in response to glucose or α -ketoisocaproate (KIC). Means \pm SEM, n = 3 independent experiments. **P<0.01, KIC versus glucose 3 mmol/l. (b) Insulin secretion analysis of neonatal islets in response to glucose. Values are means \pm SEM, n = 3. **P<0.01, glucose 20 mmol/l vs 3 mmol/l. (c) miR-375 expression analysed by RT-qPCR in fetal and neonatal islets. Means \pm SEM, n=4 islet preparations. ** p<0.01.

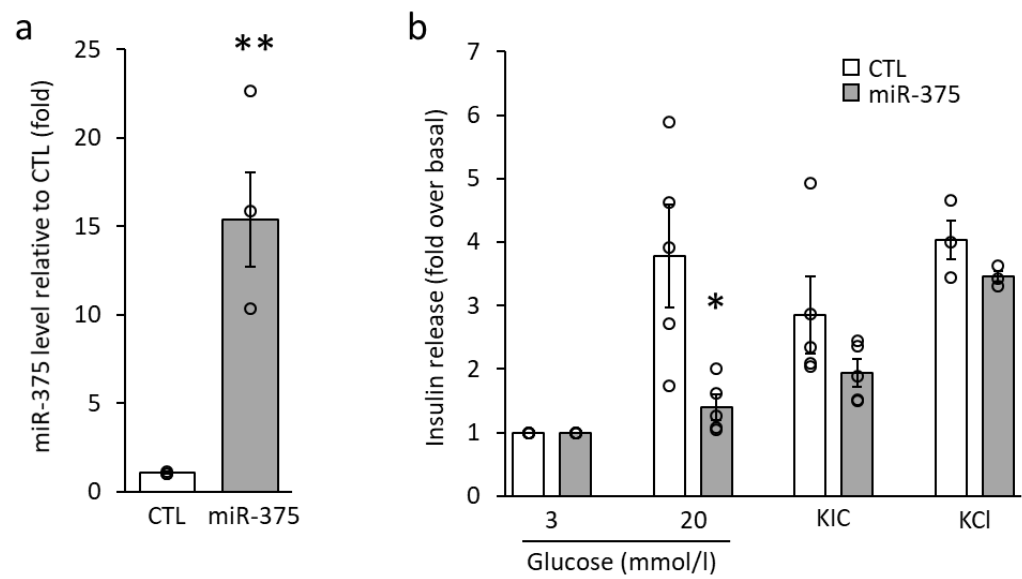








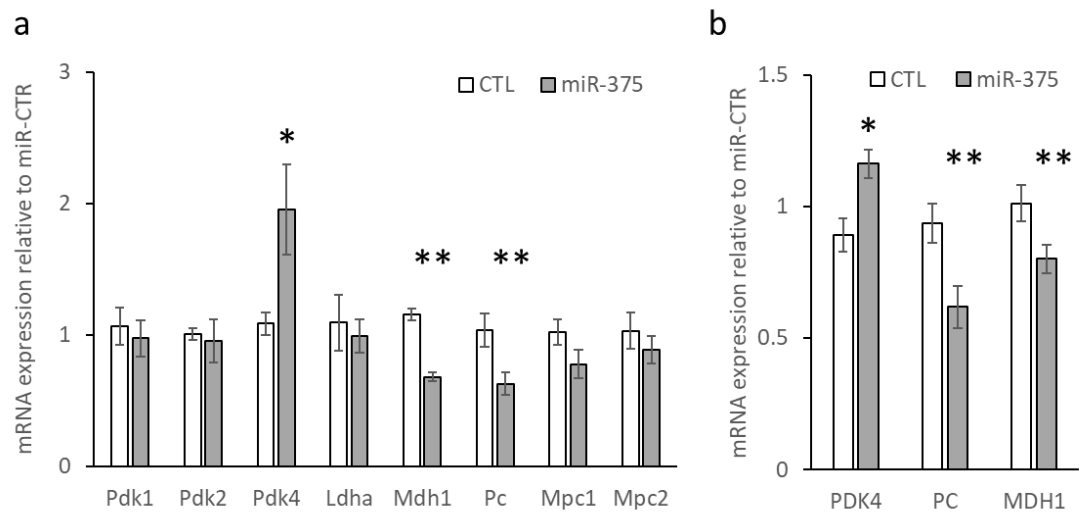
640 **Figure 5.**



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643 **Figure 6.**

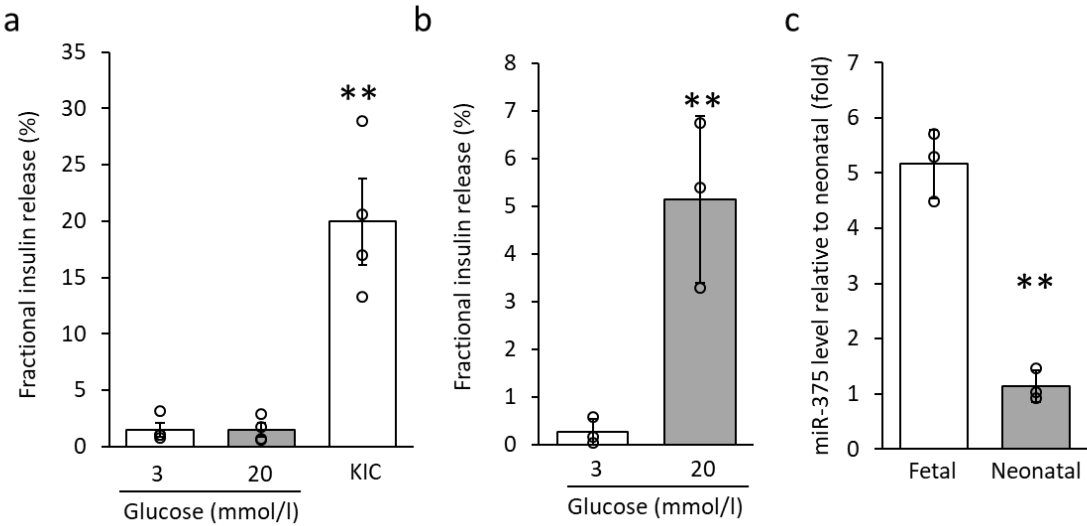


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646 **Figure 7.**

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Table 1: Sequences used for mRNA and miRNA qPCR analysis.

Human oligonucleotide sequences for qPCR analysis		
name	Reverse primer	Forward primer
Cyclophilin A	CAGTCTTGGCAGTGCAGAT	ACACGCCATAATGGCACTGG
MDH1	AGGGCACAGTCTTGCAGTTC	GCAGCTGGTCAAATTGCATA
PC	TGCCTGTCCACCAGGAACTC	GCGACTCTGTGAAACTCGCTAA
PDK4	TGGCAAGCCGTAACCAAAAC	ATGGATAATTCCCGGAATGCT
Rat oligonucleotide sequences for qPCR analysis		
name	Reverse primer	Forward primer
Cyclophilin A	CAGTCTTGGCAGTGCAGAT	ACACGCCATAATGGCACTGG
LDHA	GCGGTGATAATGACCAGCTT	ATGAGCTTGCCCTTGTTGAT
MDH1	TCACGTTGGCTTTCAGTAGG	CTGATGGAGCTGCAAGACTG
MPC1	AGCTGAGCGACTTCGTTTGT	AGATTATCAGTGGGCGGATG
MPC2	GATCCGAAACAGCTGAGAGG	TGCTGGATTAGCTGACATGG
PC	AGCCCCCTCCCAATACTCAC	CTCCCACTGCACATCCATAC
PDK1	CAGCTGTGTAAAACCGGGTA	GGATCACCCCTTCTTTGTGA
PDK2	GGTCAGGAAGCAGGTTGATCT	ACATCGAGCACTTCAGCAAG
PDK4	TGGATTGGTTGGCCTGGA	TCGCCAGAATTAAAGCTCACAC
miRNA oligonucleotide sequences for qPCR analysis		
name	oligonucleotide sequence target	
hsa-miR-375 and rno-miR-375-3p	5'UUUGUUCGUUCGGCUCGCGUGA	