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1 **microRNA-375 regulates glucose metabolism-related signaling for insulin**  
2 **secretion**

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20 **Short title.** microRNA-375 modulates insulin secretion

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

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23 **Abstract**

24 Enhanced beta cell glycolytic and oxidative metabolism are necessary for glucose-induced  
25 insulin secretion. While several microRNAs modulate beta cell homeostasis, microRNA-375  
26 stands out as it is highly expressed in beta cells where it regulates beta cell function,  
27 proliferation and differentiation. As glucose metabolism is central in all aspects of beta cell  
28 functioning, we investigated the role of microRNA-375 in this process using human and rat  
29 islets; the latter being an appropriate model for in depth investigation. We used forced  
30 expression and repression of microRNA-375 in rat and human primary islet cells followed by  
31 analysis of insulin secretion and metabolism. Additionally, microRNA-375 expression and  
32 glucose-induced insulin secretion were compared in islets from rats at different developmental  
33 ages. We found that overexpressing of microRNA-375 in rat and human islet cells blunted  
34 insulin secretion in response to glucose but not to  $\alpha$ -ketoisocaproate or KCl. Further,  
35 microRNA-375 reduced O<sub>2</sub> consumption related to glycolysis and pyruvate metabolism, but  
36 not in response to  $\alpha$ -ketoisocaproate .Concomitantly, lactate production was augmented  
37 suggesting that glucose-derived pyruvate is shifted away from mitochondria. Forced  
38 microRNA-375 expression in rat or human islets increased mRNA levels of pyruvate  
39 dehydrogenase kinase-4, but decreased those of pyruvate carboxylase and malate  
40 dehydrogenase1. Finally, reduced microRNA-375 expression was associated with maturation  
41 of fetal rat beta cells and acquisition of glucose-induced insulin secretion function. Altogether  
42 our findings identify microRNA-375 as an efficacious regulator of beta cell glucose metabolism  
43 and of insulin secretion, and could be determinant to functional beta cell developmental  
44 maturation.

45

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.

52

53

54

55

## 56 **Introduction**

57 The main beta cell mission consists in its ability to secrete insulin for maintenance of  
58 organismal glucose homeostasis. This hallmark requires expression of a set of genes (allowed  
59 genes) and the concomitant repression of others (disallowed genes) (Pullen and Rutter 2013;  
60 Quintens, et al. 2008). Indeed, beta cells have a dedicated metabolism comprised of a tightly  
61 regulated chain of elements from glucose sensors to a specialized exocytosis machinery.  
62 Glucose metabolism in beta cells linked to insulin secretion has several exclusive features. First,  
63 glucose transport is not limiting for its metabolism, and it results in a rapid equilibrium between  
64 intracellular and extracellular glucose at all glycemic levels. The second particular trait is that  
65 glycolysis is initiated by glucokinase having a high  $K_m$  for glucose associated to an elevated  
66  $V_{max}$ , and being devoid of feedback control (Prentki, et al. 2013). As beta cells express low  
67 levels of plasma membrane monocarboxylate transporters, little or no monocarboxylates,  
68 including lactate, enter the cells. Further, the low lactate dehydrogenase (LDH) activity of the  
69 beta cells accounts for the fact that a major fraction of the glucose entering the beta cells is  
70 oxidized by the mitochondria (Delghingaro-Augusto, et al. 2009; Schuit, et al. 1997). These  
71 distinctive beta cell properties must be maintained to achieve efficient oxidative  
72 phosphorylation, lipid metabolism and signaling (Prentki et al. 2013), thus making optimal  
73 glucose sensing possible. It is thought that beta cell dysfunction in type 2 diabetes could result  
74 from a reduced expression of key genes, triggering the loss of beta cell identity and hence  
75 promoting dedifferentiation (Delghingaro-Augusto et al. 2009; Talchai, et al. 2012; Weir, et al.  
76 2001). Importantly, grounds have been gleaned in favor of beta cell dedifferentiation in human  
77 type 2 diabetes. Indeed, dedifferentiated cells account for almost 40% of the beta cells in type  
78 2 diabetes patients compared to 8% in controls (Cinti, et al. 2016). Thus, it appears that the  
79 maintenance of beta cell identity throughout life is crucial for glucose homeostasis and  
80 organismal physiology.

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

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

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

98 Anti-miR-induced normalization of miR-375 expression levels in the islets of LP descendants  
99 partially restored insulin secretion and cell proliferation to levels of control animals. However,  
100 it should be noted that no consensus exists on the differential expression of miR-375 in the  
101 healthy versus the type 2 diabetic condition as reported by other studies in humans (Ofori, et al.  
102 2017; Seyhan, et al. 2016) or animals (Esguerra, et al. 2011), in which no increased miR-375  
103 expression was observed in the disease situation. This missing general agreement could be due,  
104 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

124 **Materials and methods**

125 *Reagents*

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

129

130 *Human islets*

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

138

139 *Animals and diets*

140 All procedures followed ARRIVE guidelines, were conducted in accordance with EU directives  
141 for animal experiments (2010/63/EU) and were approved by the French Research Ministry  
142 (MESR 00500.02). Three month-old Wistar rats (Janvier; Le Genest Saint Isle, France) were  
143 kept under conventional conditions, with free access to water and food. Nulliparous female rats  
144 (200–250 g) were mated with male rats and used at day 21 of gestation.

145

146 *Islet isolation*

147 3 month-old rat islets were obtained after density gradient centrifugation in histopaque-1077  
148 (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).

151

#### 152 *Culture and transfection of dissociated islet cells*

153 Rat or human islets were dissociated by trypsinization, and seeded at a density of  $25 \times 10^3$   
154 cells/cm<sup>2</sup> 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.

163

#### 164 *Cytosolic Ca<sup>2+</sup> 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).

172

#### 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  $\mu$ 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.

189

#### 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.

195

#### 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

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

204

#### 205 *Glucose uptake*

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

212

#### 213 *Mitochondrial metabolism*

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

219

#### 220 *Statistical analysis*

221 Data are presented as mean ± SEM. Statistical analyses were performed using InStat3 software  
222 (Graphpad, San Diego, CA, USA). Paired two-tailed Student's t tests were used to compare  
223 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

227 **Results**

228 *miR-375 overexpression in islet cells impairs insulin secretion and the Ca<sup>2+</sup> rise induced by*  
229 *glucose but not by KCl*

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

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

249

250 *miR-375 impacts glucose-stimulated respiration*

251 To investigate whether the dampened insulinotropic action of glucose could be due to a miR-  
252 375-induced inhibition of glucose uptake, we compared glucose transport using  $^3\text{H}$ -2-  
253 deoxyglucose in adult rat primary islet cells transfected with control microRNA or with miR-  
254 375. As shown (Fig. 2d), no difference in 2-deoxyglucose uptake was observed at 20 mmol/l  
255 glucose. To decrypt miR-375 effects on intermediate metabolism,  $\text{O}_2$  consumption in response  
256 to glucose was evaluated. It was profoundly reduced by miR-375 (Fig. 2e-g) to a similar extent  
257 as the GSIS inhibition. Moreover, miR-375 transfected cells must synthesize less ATP in  
258 response to glucose as they consume less oxygen (Fig. 2f) with an equivalent coupling to ATP  
259 synthesis (Fig. 2g).

260

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

263 To confirm the repressive miR-375 action on insulin secretion, we decreased the endogenous  
264 miR-375 level with its antisens. miR-375 reduction in adult rat primary islet cells increased  
265 GSIS with no impact on insulin content (Fig. 3a-b). Remarkably, the diminished miR-375 level  
266 augmented glucose-induced  $\text{O}_2$  consumption (Fig. 3c-d). Together, our gain and loss of function  
267 approach identifies miR-375 as a robust insulin secretion modulator which impacts glucose-  
268 induced mitochondrial metabolism.

269

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

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

276 375 overexpressing islet cells. Further, mitochondrial metabolism remained unaltered in miR-  
277 375-transfected islets in response to KIC, as reflected by O<sub>2</sub> consumption determinations (Fig.  
278 4b-c).

279

#### 280 *miR-375 modifies pyruvate metabolism*

281 The above results suggest that miR-375 could perturb insulin secretion by interfering with the  
282 production of glycolysis metabolites, in particular the last one in the pathway, pyruvate. To  
283 examine if miR-375 controls pyruvate mitochondrial metabolism we tested the effect of high  
284 pyruvate (50 mmol/l) concentrations on insulin secretion and O<sub>2</sub> consumption. We used an  
285 elevated concentration of pyruvate, as it is not an efficacious secretagogue because the plasma  
286 membrane monocarboxylate transporter-1 is poorly expressed in adult islets (Pullen, et al.  
287 2012). While pyruvate induced insulin secretion in control islets, its insulinotropic effect in  
288 miR-375 transfected islets was diminished (Fig. 4d) to a similar extent as GSIS (Fig. 1a).  
289 Forced miR-375 expression also reduced O<sub>2</sub> consumption in response to pyruvate (Fig. 4e). In  
290 differentiated beta cells, pyruvate is slightly reduced to lactate and hence most of the glucose-  
291 derived pyruvate is metabolized in the mitochondria via pyruvate dehydrogenase and  
292 anaplerotic reactions (Ainscow, et al. 2000). However, islet cells overexpressing miR-375  
293 showed a 2-fold increase in lactate release into the medium (Fig. 4f). This suggests that  
294 enhanced miR-375 expression partially redirects pyruvate metabolism from mitochondrial  
295 oxidation to anaerobic glycolysis and lactate production.

296

#### 297 *Forced miR-375 expression in human islet cells reduces insulin secretion in response to glucose* 298 *but not to KCl or to KIC*

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

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

306

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

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

325

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

328 We next explored the possibility that miR-375 could be involved in beta cell metabolic  
329 maturation for GSIS and thus interfere with the acquisition of glucose-responsiveness in the  
330 transition from fetal to neonatal beta cells. We took advantage of the finding that functional  
331 maturation of glucose stimulus-secretion coupling of fetal rat islets can be accelerated *in vitro*  
332 by glucose (Sjoholm, et al. 2000a, b). Thus, we measured the expression of miR-375 in  
333 untreated fetal islets compared with fetal islets after *in vitro* maturation by glucose (neonatal  
334 islets). As illustrated in Fig. 7a, untreated fetal islets did not respond to 20 mmol/l glucose, but  
335 they released insulin when stimulated with KIC. However, after *in vitro* maturation, they  
336 secreted insulin appropriately in response to high glucose (Fig. 7b). Remarkably, lower glucose  
337 responsiveness of insulin release by untreated fetal beta cells was associated with high miR-  
338 375 expression compared to neonatal islets (Fig. 7c). Collectively, these results suggest that  
339 repression of miR-375 expression participates to the acquisition of glucose stimulus-secretion  
340 coupling.

341

## 342 **Discussion**

343 Since their discovery, several microRNAs have been implicated in the development and  
344 function of beta cells (Dumortier et al. 2016; Guay and Regazzi 2015). Amongst these, miR-  
345 375 stands out as it is highly expressed and regulates not only beta cell development and  
346 proliferation, but also its function (El Ouaamari et al. 2008; Jafarian, et al. 2015; Kloosterman  
347 et al. 2007; Nathan et al. 2015; Poy et al. 2004; Poy et al. 2009). However, while it is accepted  
348 that miR-375 is essential for endocrine pancreas development (Avnit-Sagi et al. 2009; Joglekar  
349 et al. 2009; Kloosterman et al. 2007), its inhibitory action on insulin secretion reveals its  
350 multiple facets in beta cell physiology. Thus, deciphering miR-375 precise functions and mode  
351 of action is of considerable interest. To this end, using primary rat and human islets, we delved  
352 into the miR-375 effects on glucose metabolism, that governs insulin secretion and modulates  
353 beta cell characteristics. Overall our data support the view that miR-375 inhibits GSIS by  
354 modifying the expression of a series of genes tightly linked to metabolic signaling for GSIS ,  
355 including *Pc*, *Pdk4* and *Mdh1*. *In silico* analysis, using microRNA target prediction software,  
356 did not propose their mRNAs as potential miR-375 targets. Thus, we can assume that miR-375,  
357 by targeting one or several undefined transcripts upstream of these key metabolic enzymes may  
358 regulate beta cell function linked to insulin secretion induced by glucose.

359 Insulin secretion in immature beta cells is characterized by a poor responsiveness to glucose  
360 but a mature-like sensitivity to amino acids (Bliss and Sharp 1992; Hellerstrom and Swenne  
361 1991). In addition, immature islets consume more oxygen at low glucose compared with mature  
362 islets but their ability to increase oxygen consumption in response to high glucose is  
363 considerably lesser compared with mature islets. This clearly suggests that beta cell maturation  
364 is associated with profound metabolic changes (Stolovich-Rain et al. 2015). Transcriptomic  
365 analyses to determine the basis of poor glucose responsiveness of neonatal beta cells, compared  
366 with adult beta cells, revealed that many genes are differentially expressed, including those

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

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

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

403 Recently, the concept emerged that the reduction in beta cell mass in type 2 diabetes could  
404 reflect a loss/fading of their crucial attributes rather than increased apoptosis (Brereton, et al.  
405 2016; Jeffery and Harries 2016; Talchai et al. 2012). Considering the increased miR-375  
406 expression in islets from animals and humans with type 2 diabetes demonstrated in several  
407 studies (Bloomston et al. 2007; He et al. 2017; Poy et al. 2009; Tattikota, et al. 2014; Zhao et  
408 al. 2010) , and the current study showing that miR-375 overexpression results in reduced GSIS  
409 in human and rat islets, miR-375 appears to be a likely contributor to the fading of essential  
410 characteristics of beta cells in type 2 diabetes pathogenesis.

411

## 412 **Conclusion**

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

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

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440 the manuscript. GF, VC, NG, PL, CH, CD, MT, SD, FP and JKC researched and analyzed data.  
441 EVO and MP designed the research project, contributed to the manuscript. All authors  
442 contributed to the discussion on the manuscript and approve its final version. E.V.O is the  
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446

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567

568

569 **Figure legends.**

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

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

593 Mitochondrial O<sub>2</sub> consumption coupled to ATP synthase in presence of 20 mmol/L glucose.  
594 Means ± SEM, n = 4 (a-c) or 3 (e-g). \*P < 0.05, \*\*P < 0.01, miR-375 versus control (CTL).

595 **Figure 3. Reduced miR-375 expression in adult rat primary pancreatic islet cells increases**  
596 **glucose metabolism and insulin secretion induced by glucose.** Glucose-induced insulin  
597 secretion (a), insulin content (b) and mitochondrial O<sub>2</sub> consumption (c) were evaluated 72 h  
598 after transfection with anti-miR-375 or with anti-CTL. Panel D shows the averages of O<sub>2</sub>  
599 consumption. Means ± SEM, n = 3. \*\*P < 0.01, anti-miR-375 versus anti-CTL.

600 **Figure 4. Forced miR-375 expression in adult rat primary pancreatic islet cells impairs**  
601 **pyruvate-induced insulin secretion and pyruvate metabolism with a shift toward lactate**  
602 **production, but does not affect KIC effects.** Insulin secretion measured 72 h post-transfection  
603 in dissociated islet cells incubated with various concentrations of (a) α-ketoisocaproate (KIC)  
604 or (d) pyruvate. O<sub>2</sub> consumption was recorded from cells in presence of KIC (b-c) or pyruvate  
605 (e). (f) Lactate measurement in the incubation medium of transfected dissociated islets 4h after  
606 glucose addition (11 mmol/l). Means ± SEM of 3 (a-e) or 4 (f) independent experiments. \*P <  
607 0.05, \*\*P < 0.01, miR-375 versus CTL.

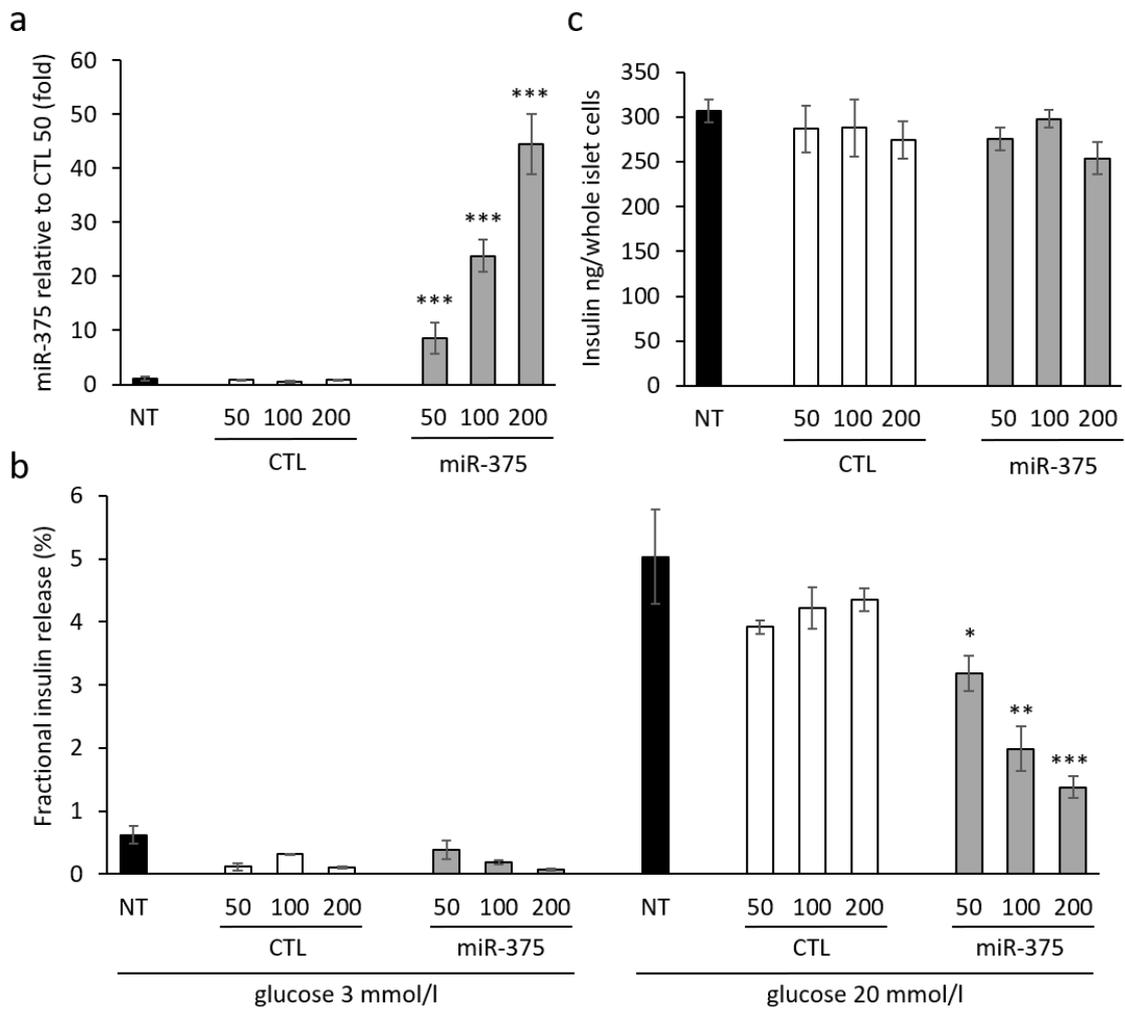
608 **Figure 5. Effect of miR-375 on insulin secretion in human islet cells.** (a) miR-375 expression  
609 analysed by qPCR in dissociated human islet cells 72 h after miR-375 transfection. (b) Insulin  
610 secretion measured in dissociated human islets 72 h after transfection and using various  
611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

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

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

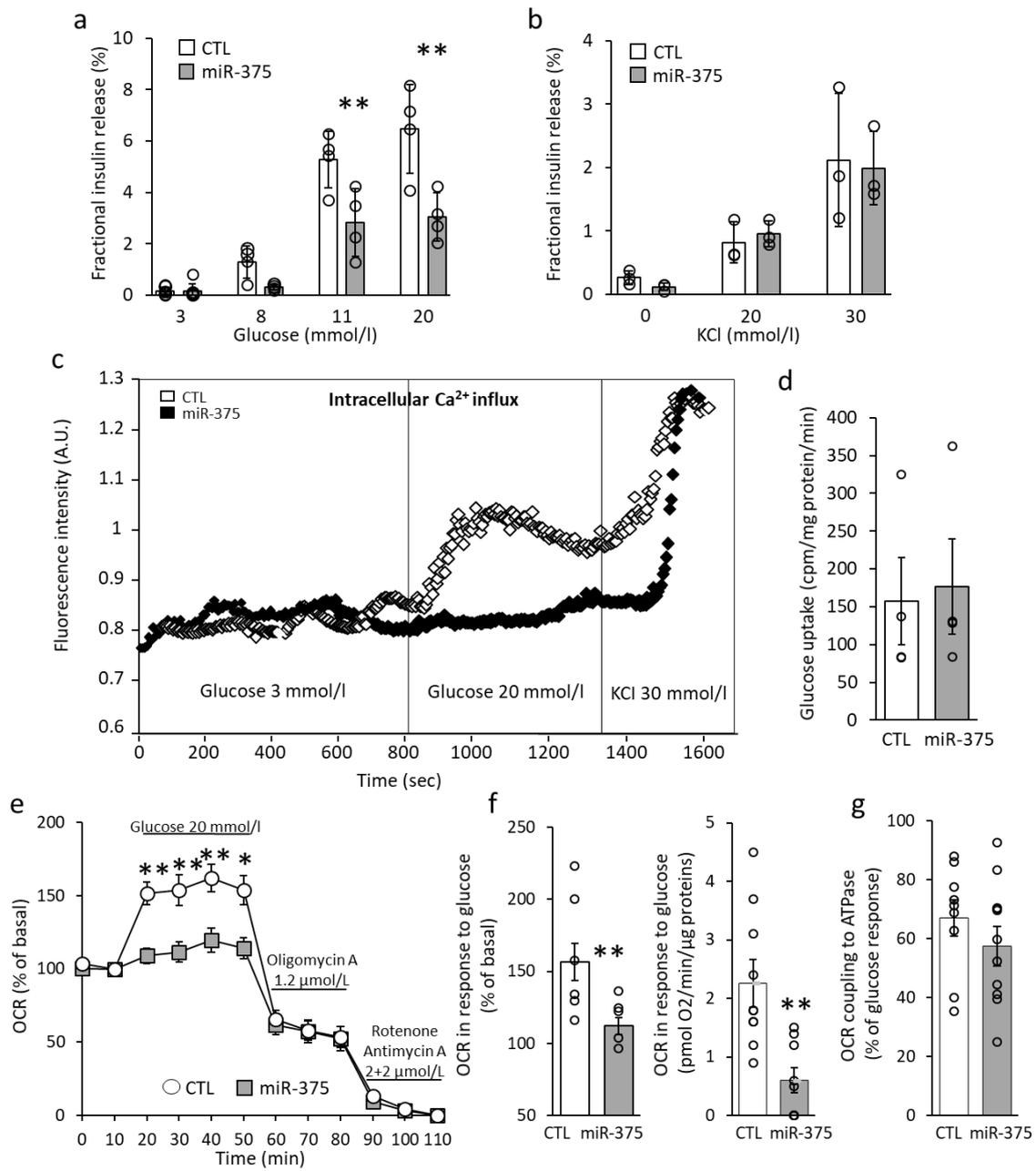
621 **Figure 7. The repression of miR-375 expression is required for the acquisition of the**  
622 **competence for glucose stimulus-secretion coupling.** After their digestion, 21 day-old fetal  
623 pancreases were either cultured for 7 days in 11 mmol/l glucose for the obtention of “neonatal”  
624 islets or purified with density-gradient for fetal islet isolation. (a) Insulin secretion analysis of  
625 fetal islets in response to glucose or  $\alpha$ -ketoisocaproate (KIC). Means  $\pm$  SEM, n = 3 independent  
626 experiments. \*\*P<0.01, KIC versus glucose 3 mmol/l. (b) Insulin secretion analysis of neonatal  
627 islets in response to glucose. Values are means  $\pm$  SEM, n = 3. \*\*P<0.01, glucose 20 mmol/l vs  
628 3 mmol/l. (c) miR-375 expression analysed by RT-qPCR in fetal and neonatal islets. Means  $\pm$   
629 SEM, n=4 islet preparations. \*\* p<0.01.

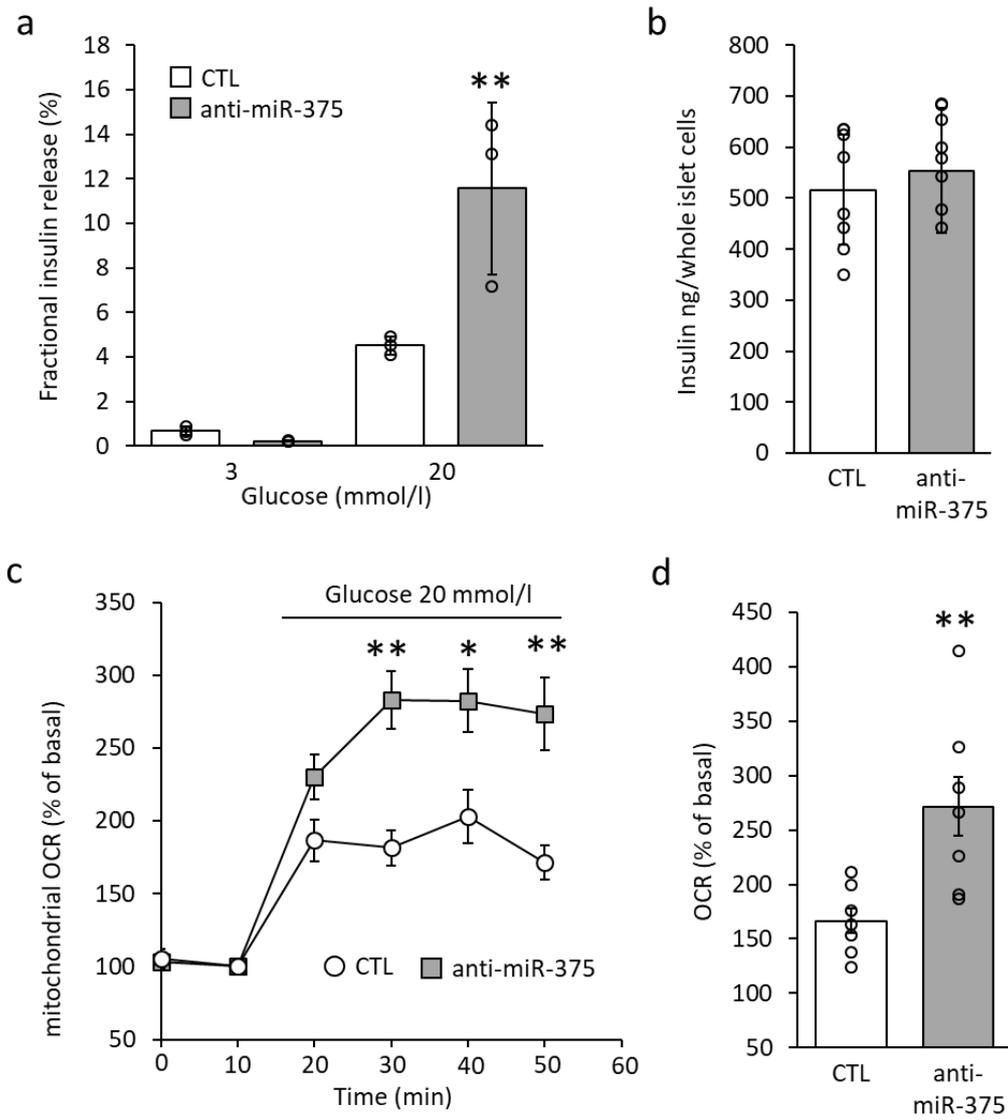
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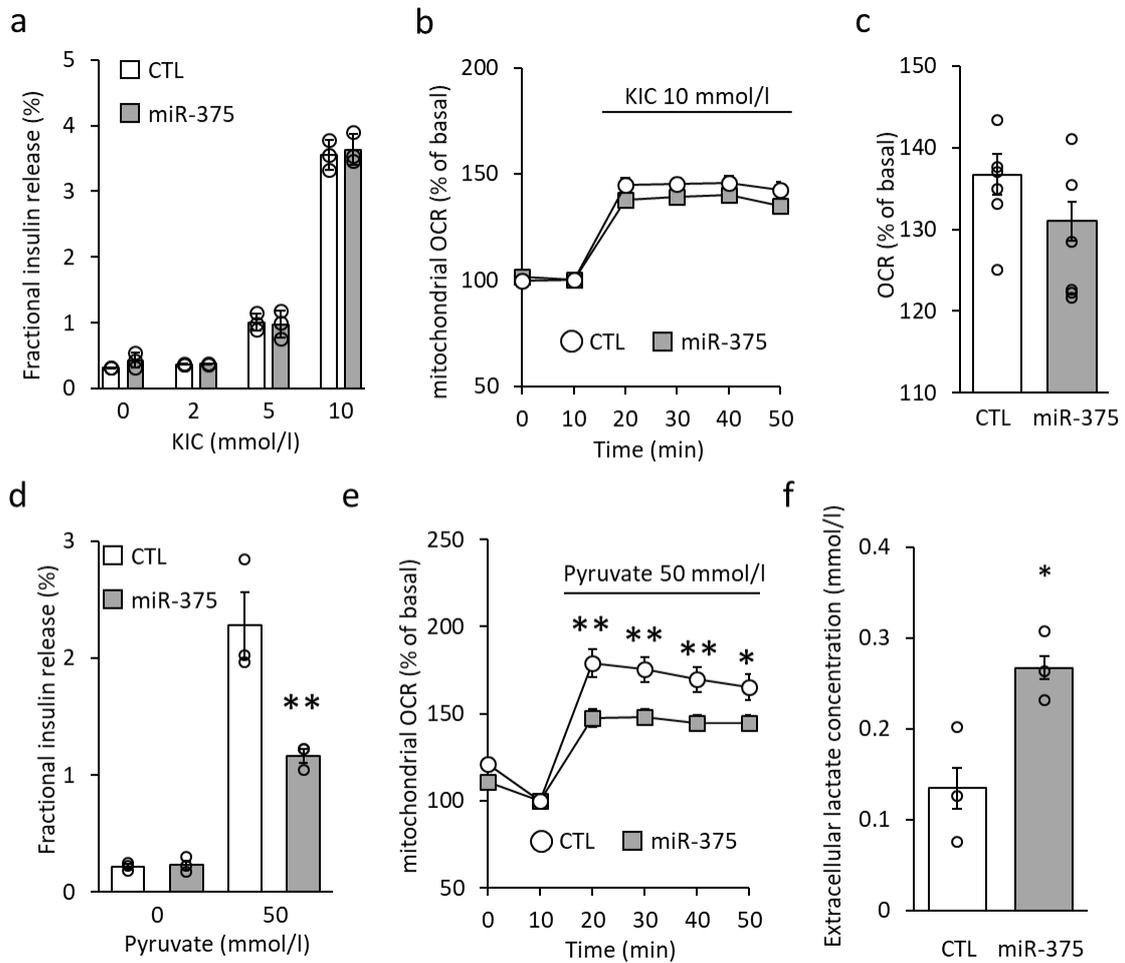


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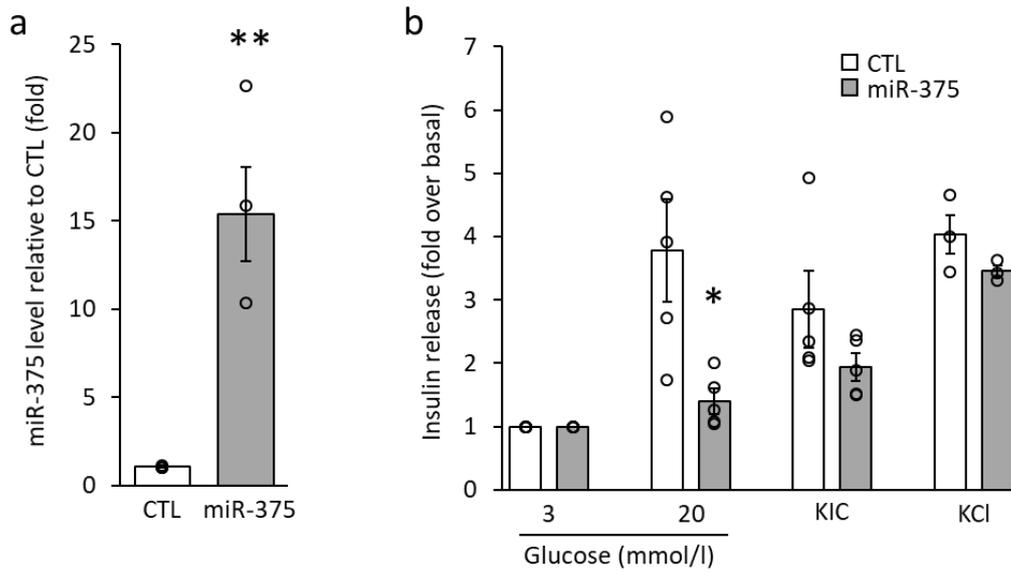
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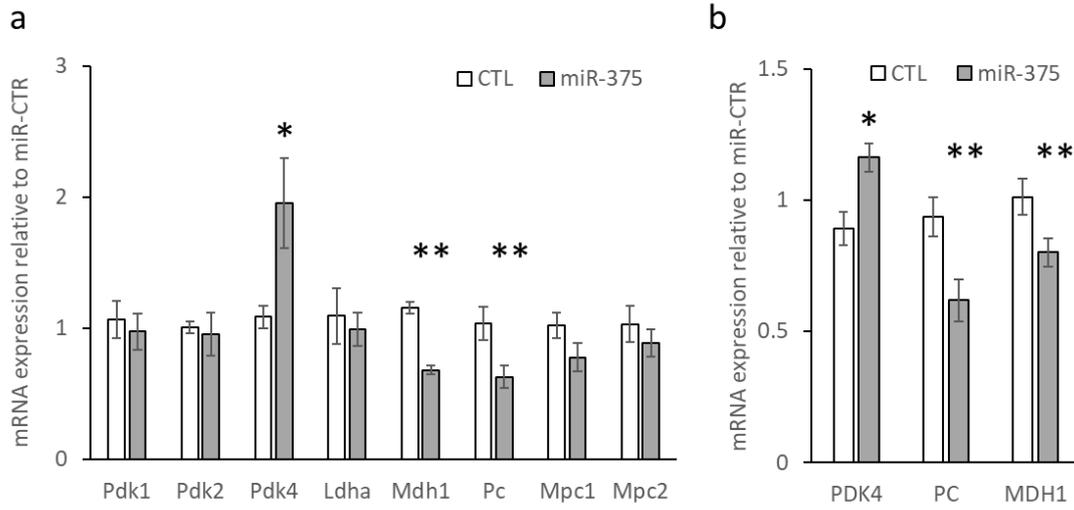
640 **Figure 5.**



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643 **Figure 6.**

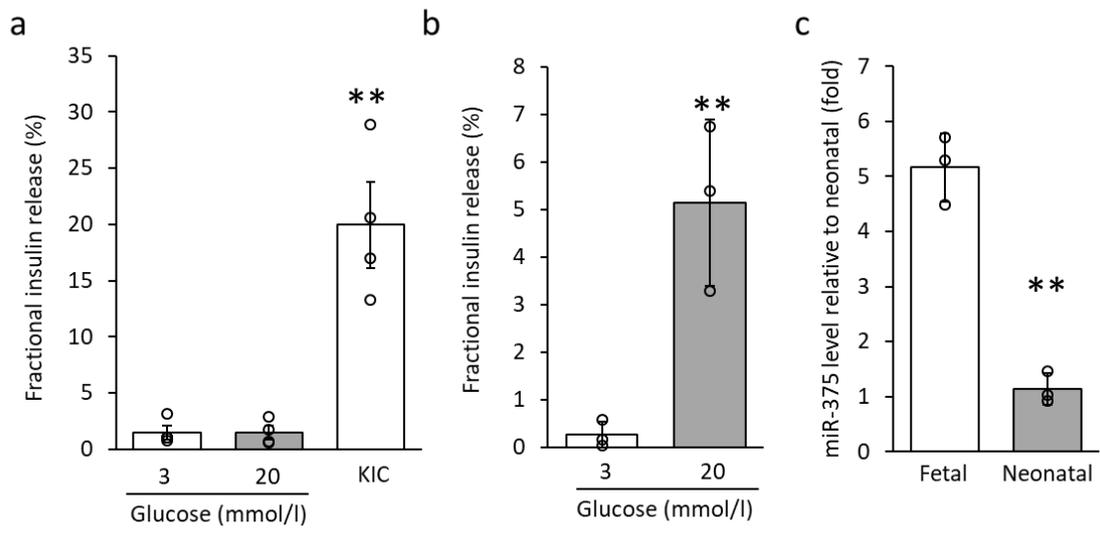


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646 **Figure 7.**

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

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