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

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Abstract

Enhanced beta cell glycolytic and oxidative metabolism are necessary for glucose-induced insulin secretion. While several microRNAs modulate beta cell homeostasis, miR-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 miR-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 miR-375 in rat and human primary islet cells followed by analysis of insulin secretion and metabolism. Additionally, miR-375 expression and glucose-induced insulin secretion were compared in islets from rats at different developmental ages. We found that overexpressing of miR-375 in rat and human islet cells blunted insulin secretion in response to glucose but not to α -ketoisocaproate or KCl. Further, miR-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 miR-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 miR-375 expression was associated with maturation of fetal rat beta cells and acquisition of glucose-induced insulin secretion function. Altogether our findings identify miR-375 as an efficacious regulator of beta cell glucose metabolism and of insulin secretion, and could be determinant to functional beta cell developmental maturation.

Key Words

- ▶ diabetes
- ▶ glucose metabolism
- ▶ insulin secretion
- ▶ microRNAs
- ▶ pancreatic islets

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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) (Quintens *et al.* 2008, Pullen & Rutter 2013). Indeed, beta cells have a dedicated metabolism comprising 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 (Schuit *et al.* 1997, Delghingaro-Augusto *et al.* 2009). 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 (Weir *et al.* 2001, Delghingaro-Augusto *et al.* 2009, Talchai *et al.* 2012). 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 microRNA in beta cells is miR-375 (Guay & Regazzi 2015, Dumortier *et al.* 2016, Martinez-Sanchez *et al.* 2016), 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 (El Ouamari *et al.* 2008, Poy *et al.* 2009, Dumortier *et al.* 2014, Latreille *et al.* 2015). In addition, several studies have found that miR-375 expression is upregulated in islets from type 2 diabetics (Bloomston *et al.* 2007, Zhao *et al.* 2010, He *et al.* 2017). 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 (Seyhan *et al.* 2016, Ofori *et al.* 2017) 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 pathophysiology, epigenetic/genetic landscape, disease progression and degree of metabolic perturbations.

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

Beta cell differentiation is a complex multistep process finely orchestrated by numerous transcription factors. The ultimate beta cell maturation step is thought

to occur around weaning. It consists in the acquisition of glucose stimulus-secretion coupling which depends on the occurrence of glucose metabolism dedicated to insulin secretion (Stolovich-Rain *et al.* 2015). As glucose metabolism governs insulin secretion and is inherent to mature beta cells we posit here that the miR-375 effects on beta cell function could be due to glucose metabolism alterations.

Materials and methods

Reagents

Culture media and buffer solutions were from Gibco (ThermoFisher Scientific), FBS and trypsin are from Invitrogen. Other reagents were from Sigma-Aldrich Chimie.

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 five adult brain-dead individuals (males=2, females=3; age, mean $\pm$ S.E.M.: 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

Three-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 RPMI-1640 medium supplemented

with FBS (10%, v/v) and antibiotics. Neoformed fetal rat islets were obtained as described (Dumortier *et al.* 2011).

Culture and transfection of dissociated islet cells

Rat or human islets were dissociated by trypsinization, and seeded at a density of 25 $\times$ 10³ cells/cm² in dishes coated with 804G-ECM (Parnaud *et al.* 2009, Dumortier *et al.* 2014). Forty-eight hours after plating, cells were transfected using Lipofectamine 2000 (Invitrogen) and with dsRNA oligonucleotides (miRIDIAN microRNA mimic, Dharmacon) corresponding to mature human and rattus miR-375-3p (ref. C-320580-01, sequence: UUUGUUCGUUCGGCUCGCGUGA) or control microRNA (ref. CN-001000-01, sequence: UCACAACCUCCUAGAAAGAGUAGA) at a concentration of 100 nmol/L. Single-stranded miRCURY LNA inhibitor (Exiqon, QIAGEN) specifically blocking endogenous miR-375-3p (ref. YI04101398) and its control (ref. YI00199006) were used at a concentration of 100 nmol/L. All analyses were performed 72 h after transfection.

Cytosolic Ca²⁺ determination

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

RNA extraction and RT-qPCR

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

For microRNA analysis, 10 ng of total RNA was reverse-transcribed using Universal cDNA synthesis kit (Exiqon). qPCR reactions were prepared using SYBR Green master mix, Universal RT (Exiqon) and microRNA LNA PCR primers (Exiqon) according to the manufacturer's manual. qPCR and data evaluation were performed using StepOnePlus apparatus and associated software (Applied Biosystem, ThermoFisher). Data were analyzed using

$\Delta\Delta C_t$ method with U6 and 5S (control primers set from Exiqon ref. YP00203906 and YP00203907) as endogenous controls and 'miR-CTL group' as control condition.

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

The primers sequences are displayed in Table 1.

Lactate determination

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

Insulin secretion

All experiments were performed using Krebs-Ringer Bicarbonate-Hepes buffer (KRBH) containing 3 mmol/L glucose and 0.1% BSA (w/v). Dissociated islet cells were preincubated for 1 h at 37°C before incubation in fresh medium containing secretagogues. After 1 h, media and cell homogenates obtained by sonification were analyzed for insulin content using a rat insulin ELISA kit (Merckodia 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 μ Ci of [3 H] deoxyglucose (Amersham Pharmacia Biotech, Uppsala, Sweden). Glucose incorporation was stopped on ice and cells were lysed using NaOH 0.2 N and neutralized with HCl 0.2 N. 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) and cultured/transfected as described earlier. The oxygen consumption rate (OCR) was determined using an XF24 Extracellular Flux Seahorse Analyzer (Agilent). Rotenone and antimycin-A (2 μ mol/L each) were used to inhibit mitochondrial respiration and oligomycin A (1.2 μ mol/L) to inhibit ATP synthase.

Statistical analysis

Data are presented as mean $\pm$ S.E.M. Statistical analyses were performed using InStat3 software (GraphPad).

Table 1 Sequences used for mRNA and microRNA qPCR analysis.

Name	Reverse primer	Forward primer
Human oligonucleotide sequences for qPCR analysis		
Cyclophilin A	CAGTCTTGGCAGTGCAGAT	ACACGCCATAATGGCACTGG
MDH1	AGGGCACAGTCTTGCAGTTC	GCAGCTGGTCAAATTGCATA
PC	TGCCTGTCCACCAGGAATC	GCGACTCTGTGAACTCGCTAA
PDK4	TGGCAAGCCGTAACCAAAAC	ATGGATAATTCCCGGAATGCT
Rat oligonucleotide sequences for qPCR analysis		
Cyclophilin A	CAGTCTTGGCAGTGCAGAT	ACACGCCATAATGGCACTGG
<i>Ldha</i>	GCGGTGATAATGACCAGCTT	ATGAGCTTGCCCTTGTTGAT
<i>Mdh1</i>	TCACGTTGGCTTTCAGTAGG	CTGATGGAGCTGCAAGACTG
<i>Mpc1</i>	AGCTGAGCGACTTCGTTTGT	AGATTATCAGTGGGCGGATG
<i>Mpc2</i>	GATCCGAAACAGCTGAGAGG	TGCTGGATTAGCTGACATGG
<i>Pc</i>	AGCCCTTCCCAATACTCAC	CTCCCACTGCACATCCATAC
<i>Pdk1</i>	CAGCTGTGTAAACCGGGTA	GGATCACCCCTCTTTGTGA
<i>Pdk2</i>	GGTCAGGAAGCAGGTTGATCT	ACATCGAGCACTTCAGCAAG
<i>Pdk4</i>	TGGATTGGTTGGCCTGGA	TCGCCAGAATTAAAGCTCACAC
Name	Oligonucleotide sequence target	
microRNA oligonucleotide sequences for qPCR analysis		
hsa-miR-375 and rno-miR-375-3p	5'UUUGUUCGUUCGGCUCGCGUGA	

Paired two-tailed Student's *t* tests were used to compare differences between samples from paired experiments. Differences between all other datasets were analyzed by one-way ANOVA with Student–Newman–Keuls *post hoc* test. *P* values <0.05 were considered significant.

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). dsRNA oligonucleotides corresponding to mature miR-375-3p or control miRNA were first tested in a dose-response experiment from 50 to 200 nmol/L and compared to the untransfected condition (Fig. 1A, B and 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

Figure 1

Forced miR-375 expression in dissociated primary adult rat islet cells reduces glucose-induced insulin secretion. 72 h 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 microRNA (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 and 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 $\pm$ S.E.M.; *n* = 3. **P* < 0.05, miR-375 vs CTL; ***P* < 0.01, miR-375 vs CTL; ****P* < 0.001, miR-375 vs 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 and 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 three 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 O_2 consumption and (F) averages of O_2 consumption determinations made in the absence or presence of 20 mmol/L glucose. (G) Mitochondrial O_2 consumption coupled to ATP synthase in presence of 20 mmol/L glucose. Means $\pm$ S.E.M., $n = 4$ (A, B, C) or 3 (E, F, G). * $P < 0.05$, ** $P < 0.01$, miR-375 vs control (CTL).

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, F and 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 antisense. miR-375 reduction in adult rat primary islet cells increased GSIS with no impact on insulin content (Fig. 3A and B). Remarkably, the diminished miR-375 level augmented glucose-induced O_2

consumption (Fig. 3C and 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 O_2 consumption determinations (Fig. 4B and C).

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 $\pm$ S.E.M., $n = 3$. ** $P < 0.01$, anti-miR-375 vs 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 the presence of KIC (B and C) or pyruvate (E). (F) Lactate measurement in the incubation medium of transfected dissociated islets 4 h after glucose addition (11 mmol/L). Means $\pm$ S.E.M. of 3 (A, B, C, D, E) or 4 (F) independent experiments. * $P < 0.05$, ** $P < 0.01$, miR-375 vs CTL.

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 five 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

Figure 5

Effect of miR-375 on insulin secretion in human islet cells. (A) miR-375 expression analyzed 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 $\pm$ s.e.m., $n = 5$ human islet isolations. * $P < 0.05$, ** $P < 0.01$, vs 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 $\pm$ s.e.m., $n = 6$ (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 vs CTL, ** $P < 0.01$, miR-375 vs CTL.

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

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 $\pm$ s.e.m., $n = 3$ independent experiments. ** $P < 0.01$, KIC vs glucose 3 mmol/L. (B) Insulin secretion analysis of neonatal islets in response to glucose. Values are means $\pm$ s.e.m., $n = 3$. ** $P < 0.01$, glucose 20 mmol/L vs 3 mmol/L. (C) miR-375 expression analyzed by RT-qPCR in fetal and neonatal islets. Means $\pm$ s.e.m., $n = 4$ islet preparations. ** $P < 0.01$.

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 in the acquisition of glucose stimulus-secretion coupling.

Discussion

Since their discovery, several microRNAs have been implicated in the development and function of beta cells (Guay & Regazzi 2015, Dumortier *et al.* 2016). Among these, miR-375 stands out as it is highly expressed and regulates not only beta cell development and proliferation, but also its function (Poy *et al.* 2004, Kloosterman *et al.* 2007, El Ouamari *et al.* 2008, Poy *et al.* 2009, Jafarian *et al.* 2015, Nathan *et al.* 2015). However, while it is accepted that miR-375 is essential for endocrine pancreas development (Kloosterman *et al.* 2007, Avnit-Sagi *et al.* 2009, Joglekar *et al.* 2009), its inhibitory action on insulin secretion reveals its multiple facets in beta cell physiology. Thus, deciphering miR-375's 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's 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 (Hellerstrom & Swenne 1991, Bliss & Sharp 1992). 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 toward 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.

In contrast to previous reports using MIN-6 insulinoma cells or primary mice β -cells (Poy *et al.* 2004, 2009, Latreille *et al.* 2015), overexpression of miR-375 has no impact on insulin exocytosis, *per se*, 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-1832/13) (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 & 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 (Talchai *et al.* 2012, Brereton *et al.* 2016, Jeffery & Harries 2016). Considering the increased miR-375 expression in islets from animals and humans with type 2 diabetes demonstrated in several studies (Bloomston *et al.* 2007, Poy *et al.* 2009, Zhao *et al.* 2010, Tattikota *et al.* 2014, He *et al.* 2017), 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 interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

O D and D F P designed the study, researched data, and contributed to the manuscript. G F, V C, N G, P L, C H, C D, M T, S D, F P and J K C researched and analyzed data. E V O and M P 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.

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