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Differences in islet-enriched miRNAs in healthy and glucose intolerant human subjects

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ABSTRACT

Many microRNAs (miRNAs) are known to be cell-type specific and are implicated in development of diseases. We investigated the global expression pattern of miRNAs in human pancreatic islets compared to liver and skeletal muscle, using bead-based technology and quantitative RT-PCR. In addition to the known islet-specific miR-375, we also found enrichment of miR-127-3p, miR-184, miR-195 and miR-493* in the pancreatic islets. The expression of miR-375, miR-127-3p, miR-184 and the liver-enriched miR-122 is positively correlated to insulin biosynthesis, while the expression of miR-127-3p and miR-184 is negatively correlated to glucose-stimulated insulin secretion (GSIS). These correlations were absent in islets of glucose intolerant donors (HbA1c $\geqslant$ 6.1). We suggest that the presence of an islet-specific miRNA network, which consists of at least miR-375, miR-127-3p and miR-184, potentially involved in insulin secretion. Our results provide new insight into miRNA-mediated regulation of insulin secretion in healthy and glucose intolerant subjects.

1. Introduction

MicroRNAs (miRNAs) are short (21–23 nucleotides) non-coding RNA molecules which regulate gene expression by binding to the 3′-untranslated regions (UTR) of messenger RNAs (mRNAs) [1], resulting in mRNA degradation, mRNA deadenylation and/or translational repression [2,3]. In recent years, a number of miRNAs have been implicated in the aetiology of several diseases including cancer [4], autoimmunity [5] and diabetes [6].

Type 2 diabetes is a complex, polygenic disease influenced by both genetic and environmental factors. It is characterized by hyperglycemia due to insulin resistance and/or impaired insulin secretion in the pancreatic β-cells. In the latter scenario, miR-375 [7,8], miR-9 [9] and miR-7 [10] have been suggested to be important regulators of insulin secretion. Over-expression of miR-375 reduces insulin secretion through inhibition of exocytosis of insulin granules via translational repression of the cytoplasmic protein myotrophin [7]. Mice lacking miR-375 (375KO) are hyperglycemic, and pancreatic β-cell mass is decreased due to impaired proliferation [8]. These studies indicate that optimal insulin secretion requires an optimal balance between the levels of miR-375 and the target proteins it controls. Indeed, the relationship between miR-375 and its target myotrophin, in particular has been suggested to be a prime example of a tuning interaction, where the miRNA acts as a rheostat, keeping the protein within a functional range of expression [11].

Although a number of miRNAs have been shown to be capable of regulating insulin secretion, and are therefore important in the development of type 2 diabetes, their expression has been poorly investigated in human glucose-sensitive tissues. In this study, we have investigated the expression of a set of miRNAs in human pancreatic islets from non-diabetic donors and compared it to (1) the expression pattern in liver and skeletal muscle to identify miRNAs important for islet function and (2) the expression pattern in islets from glucose tolerant human donors (HbA1c $\geqslant$ 6.1) to reveal abnormalities in islet miRNA expression in this group. We have correlated miRNA expression with insulin biosynthesis and GSIS, in islets from both healthy and glucose in tolerant human donors, to investigate potential regulation of insulin secretion by miRNAs. Finally, we performed computational miRNA target analysis to generate a putative target gene list for further characterization.

Abbreviations: miRNA, microRNA; GSIS, glucose-stimulated insulin secretion; KRB buffer, Krebs Ringer bicarbonate buffer.

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2. Materials and methods

2.1. Human samples

Human islets from donors ($n = 15$) were obtained through collaboration between Lund University Diabetes Centre (LUDC) and the Nordic Network for Clinical Islet Transplantation (Prof. Olle Korsgren, Uppsala University). Donors with an HbA1c < 6.1 were regarded as non-diabetic ($n = 9$), and donors with an HbA1c $\geqslant$ 6.1 as glucose intolerant ($n = 6$). Human liver ($n = 3$) and skeletal muscle RNA ($n = 2$) were commercially obtained (Ambion, Austin, TX, USA and Stratagene Agilent, Santa Clara, CA, USA).

2.2. miRNA/RNA isolation

Islets were homogenized in Qiazol buffer, and total RNA including small RNAs (smaller than 200 nt) was isolated using miRNeasy kit (Qiagen, Hilden, Germany). RNA concentration was determined using Nanodrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA), and RNA quality was determined by using the Experion Automated Electrophoresis System (BioRad Laboratories, CA, USA).

2.3. Profiling using bead-based hybridization technology

The Luminex xMAP multiplexing technology (Luminex, Austin, TX, USA) combined with Locked Nucleic Acids (LNA; Exiqon, Vedbaek, Denmark) was used to deliver highly specific profiling of 319 miRNAs. Briefly, 10 μg of total human RNA was biotinylated at the 3′-end and incubated with fluorescently labeled xMAP beads. The LNA probes on the xMAP beads were manufactured to hybridize selected miRNA sequences listed in the miRBase version 8.0. After LNA/miRNA hybridization, fresh reporter solution containing streptavidin–phycoerythrin (SA–PE) was added to the sample wells in a filter-plate (Millipore, Billerica, MA, USA). The RNA samples hybridized to the fluorescent xMAP beads were analyzed using Luminex 200. Software program Luminex IS100 version 2.3 was used for data retrieval (Luminex, Austin, TX, USA). Data were normalized for the amount of starting material and the biotin labeling grade.

2.4. Cluster analysis

The normalized bead signals were subjected to cluster analysis using Cluster 3.0 and visualized in Java TreeView (Fig. 1A). Data were presented as a heat map with the expression levels in liver and skeletal muscle relative to those in islets.

2.5. Assay validation by stem-loop RT-PCR

Quantification of miRNA levels was performed with stem-loop RT-PCR [12] following the Applied Biosystems protocol for TaqMan MicroRNA Assays with Applied Biosystems 7900 Fast Real-Time PCR system, utilizing TaqMan Universal reagents and miRNA assays (Applied Biosystems, Foster City, CA, USA). The relative quantity (RQ) determines the change in transcript expression in the human tissues. Reverse transcription (RT) and PCR were done in triplicates using stem-looped-primers, specific for each miRNA (Applied Biosystems, Foster City, CA, USA). Based on the formula $\text{A.U.} = 2^{-\Delta C_t}$, where C_t is the number of cycles at which amplification reaches a threshold, within the exponential amplification phase, miRNA C_t data were normalized to human endogenous control RNU48, assay TM1006 (Applied Biosystems, Foster City, CA, USA).

2.6. Quantitative PCR of insulin mRNA

Human insulin mRNA expression was determined by converting total RNA to cDNA utilizing Qiagen Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative PCR was performed using Applied Biosystems Hs00355773_m1 insulin assay. C_t data were normalized to human endogenous control 18S, assay Hs999999m1 (Applied Biosystems, Foster City, CA, USA).

2.7. Glucose-stimulated insulin secretion in human islets

Islets were hand-picked under a stereomicroscope at room temperature and pre-incubated for 30 min at 37 °C in Krebs Ringer bicarbonate (KRB) buffer, pH 7.4, supplemented with *N*-2 hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (10 mmol/1), 0.1% bovine serum albumin, and 1 mmol/l glucose. Each incubation vial ($n = 6$–8) contained 12 islets in 1.0 ml KRB buffer solution and was treated with 95% O_2/5% CO_2 to obtain constant pH and oxygenation. After pre-incubation, the buffer was changed to a KRB buffer containing either 1 or 16.7 mM glucose. The islets were then incubated for 1 h at 37 °C in a metabolic shaker (30 cycles per min). Immediately after incubation an aliquot of the medium was removed for analysis of insulin using a radioimmunoassay kit (Euro-Diagnostica, Malmö, Sweden). Insulin secretion index was calculated as the fold-increase in insulin release at 16.7 mM glucose compared to insulin secretion in presence of 1 mM glucose.

2.8. Target prediction and Gene Ontology analysis of islet-enriched miRNAs

Target scan (Release 5.1 April 2009) [13] was used to predict miRNA targets. The predictions cover all human genes, and to reduce the false positive hits, we filtered the target list with human islet mRNA data from the T1DBase gene atlas (http://www.t1dbase.org) [14]. To ensure that the targets are indeed actively expressed in islets, we further filtered the target list with recently published open chromatin data in the human islets consisting of ~3000 genes with islet-specific expression [15]. Targets were then tested for enrichment for Gene Ontology terms and pathways as implemented in the web-fronted DAVID Functional Annotation tool (http://david.abcc.ncifcrf.gov/) [16].

2.9. Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using students *t*-test, non-parametric Spearman rank-correlation and linear regression.

3. Results and discussion

3.1. Differential miRNA expression in human islets, liver tissue and skeletal muscle

In order to reveal miRNA expression differences in tissues with a central role in glucose metabolism, we investigated RNA samples from human islets of healthy donors, skeletal muscle and liver. As an exploratory step we used the Luminex Flexmir bead-based hybridization technology to profile global miRNA levels in the tissues. Cluster analysis of 319 miRNAs allowed the separation of islet-enriched miRNAs (from one donor) from those of liver and/or muscle-enriched miRNAs (Fig. 1A). We found six miRNAs that displayed higher expression in islets (Fig. 1A; indicated by arrows at the top part of the heat map). These include miR-493*, miR-492, miR-21, miR-521, miR-375 and miR-127-3p. As expected, the muscle specific miRNA, miR-1 [17], was more highly expressed in skeletal muscle than in liver and islets. Likewise, miR-122, earlier demonstrated to be highly abundant in the liver [18], was verified to be expressed to a higher extent in liver than in islets and skeletal muscle. In addition, miR-184 and miR-195 were found to have higher expressions in liver and muscle compared to islets.

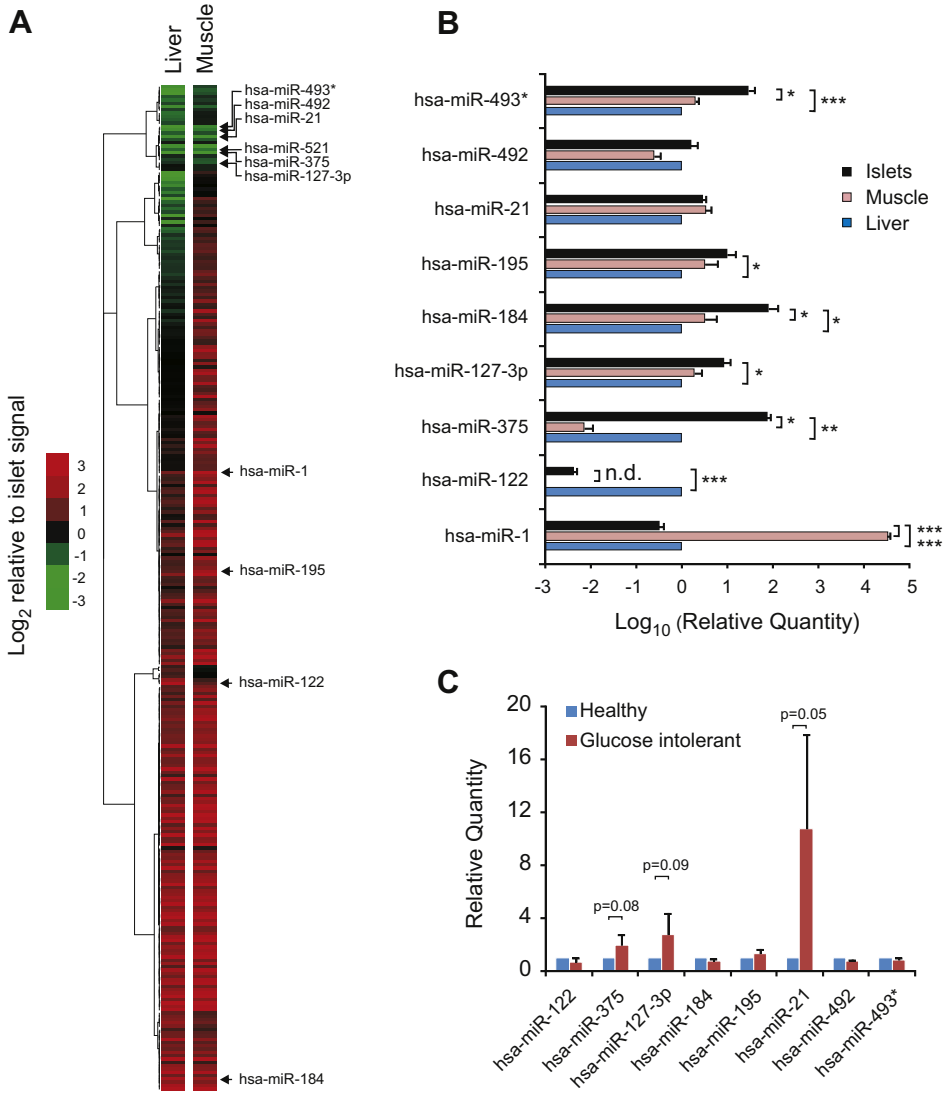

Fig. 1. Profiling of microRNA expression in human islets, liver and skeletal muscle. (A) Hierarchial clustering of array signals from 319 miRNAs included in the FlexMir miRNA profiling. The heat map presents the expression of the specific miRNAs in liver (left) and muscle (right) relative to the expression in islets. High miRNA expression levels in islets compared to muscle and/or liver are denoted in green. The 10 microRNAs chosen for RT-PCR validation are indicated by arrows. (B) Stem-loop RT-PCR validation of nine selected miRNAs in islets ($n = 9$ healthy donors) and commercial liver ($n = 3$) and skeletal muscle ($n = 2$) samples as indicated. The miRNA expression levels are presented relative to the expression in liver. (C) Comparison of miRNA expression in healthy and glucose intolerant islets measured with stem-loop RT-PCR. The analysis was performed on eight different miRNAs as indicated. The miRNA expression levels are presented relative to the expression in islets from healthy donors. Data are presented as mean ± SEM in (B) and (C). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs* expression in islets in (B).

Clustering of the relative miRNA expression in the three different tissues enabled the selection of miRNAs highly expressed in islets for validation by quantitative stem-loop PCR. For negative controls we selected miRNAs with higher expression levels in skeletal muscle and/or liver compared to islets. Ten selected microRNAs (miR-493*, miR-492, miR-21, miR-521, miR-375, miR-127-3p, miR-1, miR-122, miR-184 and miR-195) were investigated in islets from nine healthy human donors (Supplementary Table 1 and Fig. 1B), and in commercial liver and skeletal muscle RNA samples (Fig. 1B). MicroRNA-521 was excluded due to the low levels of detection in the qPCR. For the remaining nine miRNAs, the qPCR and bead-based expression data were in good agreement except for miR-184 and miR-21. Interestingly, miR-184 was found to have higher expression in islets compared to liver and muscle (Fig. 1B). Thus we considered this miRNA as enriched in islets due to the fact that qPCR has wider range of detection limit and that the result came from several donors. Meanwhile, the expression level of miR-21 was found not to be significantly different in islets compared to liver and skeletal muscle.

In summary, we were able to show the unique miRNA signature in the islets compared to those of muscle and liver, in agreement with previous observation on such tissue-specific miRNA expression patterns [7,10,17,19]. Moreover, in addition to the known islet-specific miR-375, we found miR-493*, miR-195, miR-184 and miR-127-3p to be also enriched in the pancreatic islets (Fig. 1B).

Next, we compared miRNA levels in islets from healthy and glucose intolerant donors (Fig. 1C). Islets from glucose intolerant donors showed significantly higher miR-21 expression than those from healthy donors. Likewise, the expression of miR-375 and miR-127-3p showed a tendency to be increased in the glucose intolerant donor islets compared to the healthy control islets. Of interest, miR-21 has previously been reported to be induced in human islets during inflammatory conditions and to be up-regulated in non-obese diabetic (NOD) mice [20]. Moreover, it has been

demonstrated that over-expression of miR-375 results in reduced insulin secretion [7]. Thus, the increased levels of these miRNAs potentially contribute to the impaired insulin secretion observed in hyperglycemia.

3.2. Specific miRNAs in human islets correlate to insulin biosynthesis and insulin secretion

We wanted to investigate whether the expression of specific miRNAs can influence β-cell function. Therefore we measured insulin mRNA levels in human islets (as a crude estimate for insulin biosynthesis) and GSIS in batch-incubated islets from the same donors. Insulin expression and secretion in islets from healthy donors were correlated to the expression of all nine miRNAs determined by qPCR. The expression of miR-375, miR-122, miR-184 and miR-127-3p correlated positively to insulin mRNA expression (Fig. 2A–D), whereas the other miRNAs investigated showed no significant correlation with insulin biosynthesis (data not shown). The positive correlation indicates that these miRNAs could suppress target protein(s) that normally reduce insulin biosynthesis. It might be surprising that the liver-specific miRNA miR-122 was found to be correlated to insulin biosynthesis and has the same

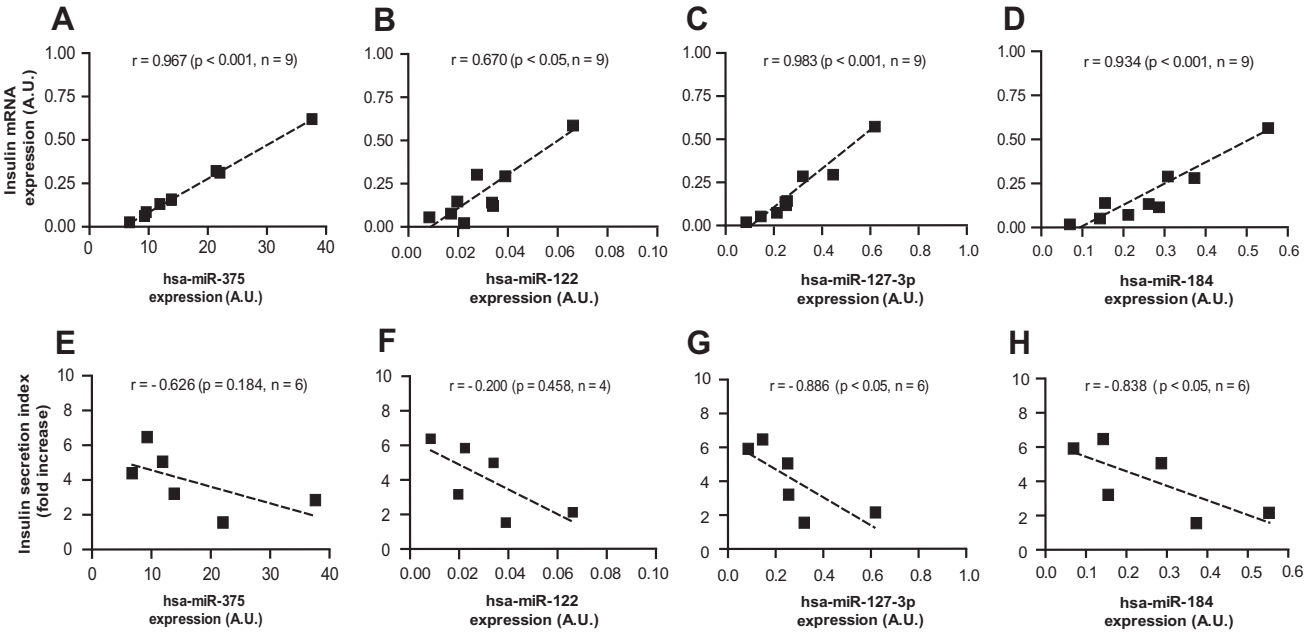

Fig. 2. Correlation between human islet miRNA expression and β-cell function. (A–D) Insulin mRNA expression measured in islets from healthy donors plotted against the indicated miRNA expression in islets from the same donors. (E–H) Insulin secretion index (fold increase in insulin secretion at 16.7 mM glucose compared with basal insulin release at 1 mM glucose) plotted against the indicated miRNA expression. Insulin secretion and miRNA expression was measured in islet from the same healthy control subjects. Spearman correlation factor (r), number of donors (n) and P-value is displayed in the upper right of each graph.

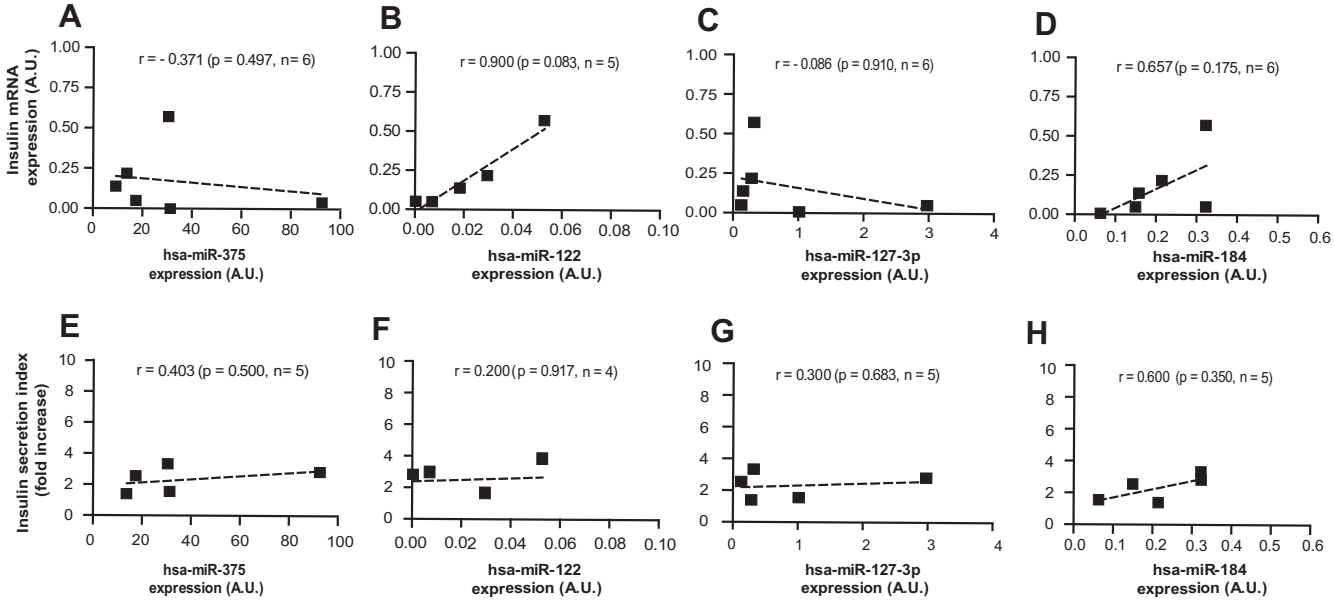

Fig. 3. β-Cell function and miRNA expression in islets from glucose intolerant donors. As in Fig. 2 but miRNA expression, insulin mRNA expression and insulin secretion was measured on islets from glucose intolerant donors. Notice the lack of correlation in islets from the glucose intolerant donors.

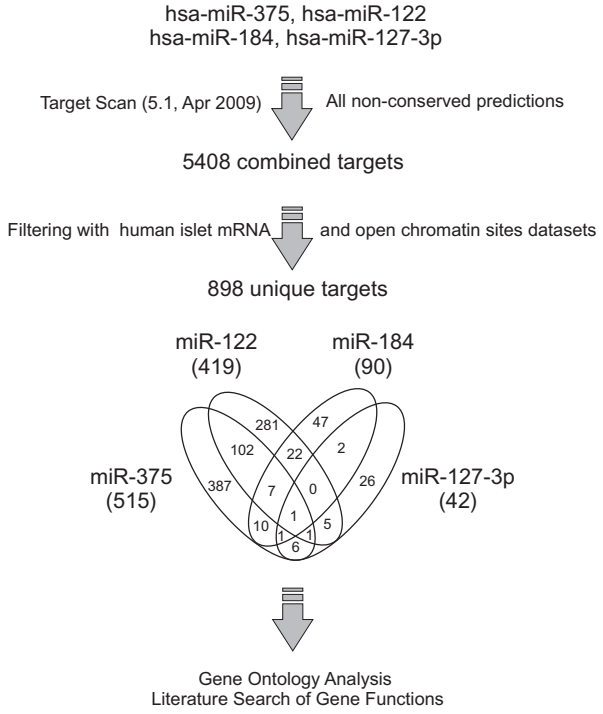

Fig. 4. Workflow of miRNA target analysis. Target analysis was performed on miRNAs with strong correlation to β-cell functions (miR-375, miR-122, miR-127-3p and miR-184). Predicted targets (5408) were filtered using available human islet mRNA data (T1DBbase) and human islet FAIRE data [15] prior to Gene Ontology Enrichment. Venn diagram of filtered mRNA targets (898) depicts common and individual targets for each of the four miRNAs.

pattern as the islet miRNA miR-375. However, earlier studies have reported a similar developmental origin for hepatocytes and pancreatic β-cells [21], suggesting that some processes may be controlled by the same miRNAs in these cell types. In addition to the positive correlation to insulin mRNA, expression of miR-184 and miR-127-3p showed a significant negative correlation to insulin secretion index (Fig. 2G and H). These results imply that these two miRNAs could repress target proteins at multiple levels of β-cell function, such as: (i) proteins involved in negative regulation of insulin biosynthesis and (ii) proteins which are important for optimal insulin secretion.

Table 1
Top three significantly-enriched Gene Ontology (GO) and KEGG Pathway categories.

	Gene count	*P*-value
Metabolic function		
GO:0008066~glutamate receptor activity	7	0.004
GO:0005509~calcium ion binding	64	0.006
GO:0004672~protein kinase activity	44	0.012
KEGG pathway		
hsa04360:Axon guidance	15	0.005
hsa04020:Calcium signaling pathway	17	0.014
hsa00512:O-Glycan biosynthesis	6	0.015

3.3. Correlation between specific miRNA expression and β-cell function is lacking in glucose intolerant donor islets

We next examined the relationship between miRNA expression and β-cell function in islets from six glucose intolerant donors. Interestingly, the correlation between insulin mRNA and the individual expression of miR-375, miR-122, miR-127-3p and miR-184 was absent in islets from glucose intolerant donors (Fig. 3A–D). Likewise, there was no correlation with GSIS (Fig. 3E–H). It has been suggested that miRNAs act as rheostats maintaining protein level output at an optimal level [11], with miR-375 as an excellent example. Indeed, our experiments indicate a perturbed regulation between miRNA expression and β-cell function not only for miR-375 but also for miR-122, miR-184 and miR-127-3p. This might be a reflection of an impaired miRNA network in patients with type 2 diabetes.

3.4. Target predictions of islet-specific miRNAs

We next searched for possible protein targets of miR-375, miR-122, miR-127-3p and miR-184 to find pathways relevant for β-cell function controlled by these miRNAs. MicroRNA targets were

Table 2
Putative gene targets of miRNAs previously associated with diabetes and/or islet functions. X denotes the presence of miRNA putative binding site in the 3′UTR of the gene.

Entrez ID	Gene symbol	hsa-miR 375	hsa-miR 122	hsa-miR 184	hsa-miR 127-3p	Annotation
3480	IGF-1R	X	X	X	X	IGF-1 receptor; important for β-cell growth and function [24,25]
3651	PDX-1		X			β-Cell specific transcription factor regulating insulin gene transcription [22]
3375	IAPP		X			Coexpressed and cosecreted with insulin by pancreatic β-cells [23]
5080	PAX6	X				Regulation of islet function in humans [26]
2740	GLP-1R		X			Variation in GLP-1R may alter insulin secretion in response to exogenous GLP-1 [27]
5362	PLXNA2	X	X	X	X	Plexin-A2; role in neuronal migration [28]
60468	BACH2	X	X			T1D risk loci [29]
169792	GLIS3	X				Defects in GLIS3 are a cause of NDH syndrome, neonatal diabetes mellitus with congenital hypothyroidism [30]
169026	SLC30A8		X			Zinc transporter 8, ZnT8; susceptibility locus diabetes mellitus [31]
6334	SCN8A	X			X	Na^+-channel, NaV 1.6; expressed in human islets [32]
775	CACNA1C		X	X		L-type voltage-gated Ca^{2+} channel present in human pancreatic β-cells; role in insulin secretion [32]
8912	CACNA1H			X		T-type voltage-gated Ca^{2+} channel present in human pancreatic β-cells; role in insulin secretion [35]
2557	GABRA4	X				$GABA_A$-receptor, alpha 4 subunit; low levels found in human islets [33]
2558	GABRA5		X			$GABA_A$-receptor, alpha 5 subunit; non-detected in human islets [33]
2565	GABRG1	X				$GABA_A$-receptor, gamma subunit; low levels found in human islets [33]
10243	GPHN	X				Scaffolding protein for GABA(A) receptors; detected in human islets, rat islets, INS-1 cells [34]
4684	NCAM1	X	X			Involved in reorganization of the F-actin network in β-cells [35]
999	RIMS1	X	X			Exocytotic protein interacting with Rab3a [36]
9341	VAMP3		X	X		Cellubrevin; involved in exocytosis [37]

predicted using Target Scan (Release 5.1 April 2009) [13]. To minimize false positive hits among the putative targets our analysis of predicted miRNA targets consisted of a series of filtering steps with published high-quality experimental data (Fig. 4).

Performing pathway analysis of all miRNA targets of any of the four miRNAs (in total 898 unique targets) revealed significant enrichment in specific Gene Ontology (GO)-terms for metabolic function and in the KEGG Pathways, "Axon guidance" and "Calcium signaling pathway" (Table 1). Examining specific target genes (Table 2) revealed genes coding for proteins known to be involved in β-cell function, such as voltage sensitive T-type (*CACNA1H*) and L-type (*CACNA1C*) Ca^{2+}-channels, several GABA-receptor subunits (*GABRA4*, *GABRA5* and *GABRG1*) and a voltage sensitive Na^{+} channel (*SCN8A*). All these targets are essential for insulin secretion and are assumed to be suppressed by increased miRNA expression. Interestingly we found that the miRNA expression levels of these four miRNAs were negatively-correlated to insulin secretion. Of interest, among the predicted target proteins are also PDX-1, IAPP and IGF-1R. PDX-1 is documented to control insulin gene expression [22] and IAPP is reported to be overexpressed in type 2 diabetes patients [23]. The IGF-1 receptor is important in the control of β-cell growth and function and mice lacking this receptor show deficient GSIS [24]. The IGF-1 receptor has also been suggested to play a crucial role in GLP-1 induced protection of β-cell apoptosis [25]. Our *in silico* analyses yielded interesting target proteins that will be valuable in the future to investigate more specifically the importance of miRNAs in the control of human β-cell function.

3.5. Final conclusion and future perspective

We have demonstrated the presence of four miRNAs (miR-375, miR-122, miR-127-3p and miR-184) in human pancreatic islets of interest for β-cell function. Based on our correlation data we hypothesize that they are of special importance for the control of insulin biosynthesis and secretion. In addition, we observed a deranged association between these miRNAs and β-cell function in islets from glucose intolerant human donors. These findings open new avenues of research aimed at exploring potential roles of these miRNAs in the maintenance of functional islet β-cells. Eventually, identification of tissue-specific miRNAs implicated in type 2 diabetes might be suitable as biomarkers and a future clinical strategy for diagnosis of the disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.024.

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