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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.