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Quantitative differential expression analysis reveals miR-7 as major islet microRNA

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Abstract

MicroRNAs (miRNAs) are non-coding gene products that regulate gene expression through specific binding to target mRNAs. Cell-specific patterns of miRNAs are associated with the acquisition and maintenance of a given phenotype, such as endocrine pancreas (islets). We hypothesized that a subset of miRNAs could be differentially expressed in the islets. Using miRNA microarray technology and quantitative RT-PCR we identified a subset of miRNAs that are the most differentially expressed islet miRNAs (ratio islet/acinar > 150-fold), miR-7 being the most abundant. A similarly high ratio for miR-7 was observed in human islets. The ratio islet/acinar for miR-375, a previously described islet miRNA, was <10 and is 2.5× more abundant in the islets than miR-7. Therefore, we conclude that miR-7 is the most abundant endocrine miRNA in islets while miR-375 is the most abundant intra-islet miRNA. Our results may offer new insights into regulatory pathways of islet gene expression.

Keywords: Pancreatic islets; MicroRNAs; MicroRNA microarrays; q-RT-PCR

MicroRNAs (miRNAs) are non-coding small RNAs (~19–22 nt) that regulate gene expression by post-transcriptional interference with specific messenger RNAs (mRNA) [1]. The discovery of miRNAs as modifiers of gene expression is one of the milestones in the field of gene expression regulation. The current knowledge of miRNA biology is mostly derived from pioneering studies performed in the nematode *Caenorhabditis elegans*, showing a temporal-specific expression of the lin-4 miRNA [2]. As each miRNA can have multiple target messenger RNAs individually or jointly with other miRNAs, they are able to control highly complex regulatory networks of gene expression [3]. The current number of human miRNA genes listed in the Sanger database is 533 [4]. It was suggested that this number will increase up to 1000, which would amount to almost 3% of the protein-coding genes [1]. However, a new mammalian miRNA atlas describes 300,000 sequences from 256 small RNA libraries and presents evidence for expression of approximately 400 miRNA genes in each genome [5].

The biological function of most miRNAs remains currently unknown, but some of their messenger RNA targets have been experimentally confirmed. To this group belongs miR-375, which negatively controls insulin secretion in β-cells by targeting myotrophin (Mtpn) [6], a gene originally described in neuron vesicle transport. Another miRNA with reported biological function in insulin producing cells is miR-9. This miRNA, predominantly expressed in brain, negatively controls insulin secretion in insulinoma cells [7].

Several studies have shown that miRNAs regulate embryonic development and have tissue/cell-specific patterns [8–11]. miRNAs are necessary for islet cell genesis in the mouse [12]. It has been reported that inhibition

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of miR-375 has a profound deleterious effect on pancreatic development, particularly on endocrine cells [13]. Thus, expression of a specific subset of miRNAs could have a crucial effect on the acquisition and maintenance of a given phenotype, such as that of pancreatic islets. For this reason, we investigated the expression profile of miRNAs differentially expressed in the endocrine pancreas (islets).

Materials and methods

Tissue procurement. Rat islets and acinar tissue were isolated at the Preclinical Cell Processing and Translational Models Core of the Diabetes Research Institute from the pancreas of donor Lewis rats (Charles River Labs) by digestion of the pancreatic tissue with purified enzyme blend Liberase RI® (Roche Molecular Biochemical, Indianapolis, IN) at a concentration of 0.16 mg/mL, following a procedure described earlier [14]. Human pancreatic islets and acinar tissue were obtained from the Human Islet Cell Processing Facility at the Diabetes Research Institute. All animal studies were performed under protocols approved by the University of Miami Animal Care and Use Committee.

MicroRNA microarray studies. Total RNA was isolated by a method that preserves RNA molecules <200 bp, using mirVana miRNA Isolation kit (Ambion, Foster City, CA). In order to achieve comparative results for all the samples, special caution was taken to prevent degradation of miRNAs and their cDNAs in RNA preparations [15]. Samples were processed for miRNA profiling by LC Sciences (Houston, TX). Two to five microgram total RNA samples were size fractionated using YM-100 Microcon centrifugal filter (Millipore, Billerica, MA) to enrich the miRNA fraction. RNA was 3′ extended with poly(A) tail. An oligonucleotide tag was then ligated to the poly(A) tail for subsequent fluorescent dye staining. Two different tags were used for the two RNA samples, islets and acinar tissue, on each dual-sample chip. Hybridization was performed overnight on a μParaFlo™ microfluidic chip using a micro-circulation pump. The Array contained probes for 312 mature microRNAs (Sanger v. 8.1) plus multiple controls including housekeeping small RNA genes and the oligonucleotide microRNA probes with single mutations (mismatch probe negative controls). Hybridization was carried out with 100 μL of 6× SSPE buffer (0.90 M NaCl, 60 mM Na_2HPO_4, and 6 mM EDTA, pH 6.8) containing 25% formamide at 34 °C. After hybridization, fluorescence labeling with tag-specific Cy3 and Cy5 dyes provided detection. For each pair of islets and acinar samples, two chips were analyzed: in one chip, islets were labeled with Cy5 and acinar with Cy3 and in the other one the dyes were reversed. Hybridization images were collected with GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, CA) and digitized by Array-Pro image analysis software (Media Cybernetics, Silver Spring, MD). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally weighted Regression) [16]. A total of three experiments were performed. Replicate hybridizations of the same islet and acinar samples were performed utilizing the two-color (Cy3/Cy5) "dye flip reversal method" (a total of six hybridizations). For two-color experiments, the ratio of the two sets of detected signals ($\log_2$ transformed, balanced) and *p*-values of the *t*-test were calculated; differentially detected signals were those with less than 0.01 *p*-values.

Statistical analysis. The averages of both hybridizations (Cy5/Cy3 and Cy3/Cy5) for the three samples were analyzed by Significant Analysis of Microarray SAM (False Discovery Rate [FDR] < 1%) [17]. The analysis only included those miRNAs detected in at least two averages arrays.

miRNA quantification: quantitative real time PCR (qRT-PCR). RNA was isolated using mirVana miRNA Isolation kit (Ambion-Applied Biosystems, Foster City, CA). Purity and concentration of the samples were assessed with a NanoDrop ND-1000 Spectrophotometer. The quantification of miRNA was performed with a method termed looped-primer RT-PCR [18], following the Applied Biosystems protocol for TaqMan MicroRNA Assays with 7500 Fast Real-Time PCR system, utilizing TaqMan Universal reagents and miRNA assays (Applied Biosystems). RQ determines the change in transcript expression in islet versus acinar tissue. RT and PCR were done in triplicates using looped-primers and primers specific for each miRNA (Applied Biosystems). RQ was calculated via Applied Biosystems SDS software based on the equation $RQ = 2^{-\Delta\Delta Ct}$, where C_t is the number of cycles at which amplification reaches a threshold, determined by the software, within the exponential amplification phase. C_t data were normalized to endogenous control miR-16.

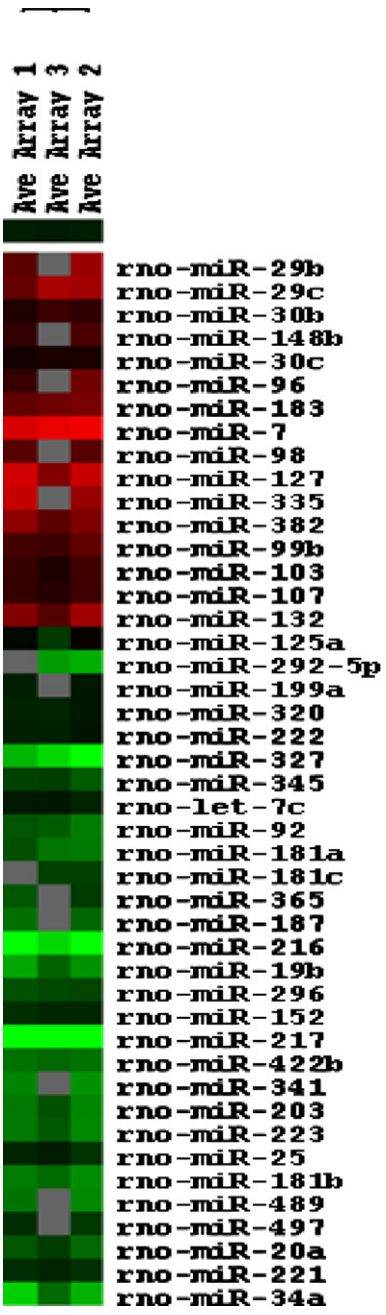

Fig. 1. Differential expression of pancreatic miRNAs. The heat map figure was obtained by Treeview [21] using the data presented from Table 1. Red represents differentially expressed islet miRNAs. Green represents differentially expressed acinar miRNAs. Colorgram depicts expression from high (bright red or green) to low (black) and no expression (gray).