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Expression of miR-206 in human islets and its role in glucokinase regulation

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Keywords: human islets, glucokinase, miR-206, diabetes
Abbreviations: T2D type 2 diabetes, GCK glucokinase, miRNA microRNA
Abstract

Inappropriate insulin secretion from β-cells is considered as an early sign of impaired glucose tolerance and Type 2 diabetes (T2D). Glucokinase (GCK) is an important enzyme that regulates glucose metabolism and ensures that the normal circulating glucose concentrations are maintained. GCK expression is induced by glucose and regulated via transcription factors and regulatory proteins. Recently, microRNA-206 (miR-206) was reported to regulate GCK and alter glucose tolerance in normal and high fat diet-fed mice. Although the study findings have implications for human diabetes, studies in human islets are lacking. Here, we analyze human islets from individuals without or with Type 2 diabetes (T2D), using TaqMan-based real-time qPCR at the tissue (isolated islet) level as well as at single-cell resolution, in order to assess the relationship between miR-206 and GCK expression in normal and T2D human islets. Our data suggest that unlike mouse islets, human islets do not exhibit any correlation between miR-206 and GCK transcripts. These data implicate the need for further studies aimed towards exploring its potential role(s) in human islets.
Introduction

The prevalence as well as incidence of Type 2 diabetes (T2D) is increasing globally. Hyperglycemia is a hallmark feature of T2D and it mainly results from insulin resistance in peripheral tissues as well as defects in pancreatic β-cell function. It is suggested that impaired β-cell function probably occurs before peripheral insulin resistance ensues in T2D (4) and that reduced first-phase insulin secretion precedes glucose intolerance and diabetes in β-cell-specific insulin receptor knock-out (KO) mice (11). Similarly, early insulin response to glucose was found to be reduced in individuals with impaired glucose tolerance (15). These studies mark the importance of glucose-mediated insulin release from β-cells as an essential step in maintaining normal glucose homeostasis and any alteration to this process is a precursor to later development of insulin intolerance and T2D.

Glucokinase (GCK) functions as the glucose sensor in β-cells by regulating the rate of glucose entry into the glycolytic pathway (13). Glucose entry and metabolism in pancreatic islet β-cells leads to insulin secretion via an intricate mechanism involving membrane depolarization and calcium ion facilitated insulin granule exocytosis. GCK, also known as hexokinase IV, converts glucose to glucose-6-phosphate (G6P). It has a low affinity for glucose and does not demonstrate end-product (G6P) feedback inhibition (23), ensuring a high threshold for glucose (around 5mM), required to secrete insulin from β-cells (12, 17). Inactivating mutation in GCK cause maturity-onset diabetes of the young/MODY2 (20); a form of monogenic T2D, while activating mutations result in congenital hyperinsulinemia (5, 19).

Glucokinase production is regulated in pancreatic β-cells by transcription factors and regulatory proteins (7, 14, 18). Recently, microRNA-206 knockout (KO) mice (21) demonstrated better glucose-stimulated insulin secretion than wild type counterparts under chow-fed as well as high fat diet (HFD)-fed conditions (21). Reporter-gene analyses pointed to a target site of miR-206 in the murine gck UTR and islet gck activity was increased in KO mice (21). This interesting study by Vinod et al (21) demonstrated post-transcriptional regulation of gck mRNA in mouse pancreatic islets by miR-206. While data in mice suggest a role of miR-206 for whole body control of glucose homeostasis, possibly via gck, the expression and regulation of miR-206 in human islets was not investigated (21). In our current study, we have measured miR-206 expression in human islets from non-diabetic as well as Type 2 diabetic donors, and further compared miR-206 levels with the expression levels of
Our analyses in whole islets as well as single cells demonstrate that GCK transcripts are not regulated by miR-206 in human islet cells.

Materials and methods

Human tissues: Isolated human islets were obtained from the Tom Mandel Islet Isolation Program at St. Vincent’s Institute, Melbourne. Diabetes was classified based on reporting by the consenting family member and/or any available clinical records. Human smooth muscle tissue samples were obtained from cholecystectomy procedures and used for this study following informed consent from patients at the Strathfield Private Hospital, Sydney. All cell/tissue samples analyzed and presented in this study have been approved by the human ethics committee at Sydney Local Health District.

RNA isolation and PCR: Tissues were collected in Trizol and RNA was extracted following the manufacturer’s instructions. Hexamer cDNA was prepared using a high capacity cDNA reverse transcription (RT) kit and quantitative real-time PCR of samples for GCK mRNA levels was performed in 96-well optical clear plates using Assay-on-Demand probe and primer mix (Table 1) with TaqMan fast universal PCR master mix. PCR was performed using the ViiA7 Real-Time PCR platform and data analyzed using Applied Biosystems ViiA7 Software. The results were normalized to the housekeeping gene (18S rRNA) and presented as fold over detectable (FoD) using methodologies published earlier (6). For miRNA detection, RNA was reverse transcribed using mature miRNA-specific RT primers and miRNA RT kit. PCR was carried out using the miRNA-specific cDNA and TaqMan qPCR primers-probe mix on an OpenArray (OA) platform (3). Data were analyzed using Thermo Cloud computing software. The results were normalized to the global mean and are presented as FoD values. All reagents for TaqMan-based real-time quantitative PCR for gene and microRNA transcripts were obtained from Thermo Fisher Scientific, Waltham, MA, USA.

Single cell PCR: Single cell PCR was carried out using protocols described earlier (10). Briefly, single cells were picked up by manual micromanipulation (pipettes/capillaries) and placed directly into Trizol to a final volume of 20 μL and re-suspended several times. Since single cell PCR involves the handling of a very low amount of RNA, we used 1 μL (1 mg/ml) nuclease-free glycogen as a carrier for RNA isolation. Following isolation, resulting RNA was re-suspended in nuclease-free water and half of it (by volume) was immediately processed to obtain cDNA using high capacity cDNA RT Kit
that includes hexamers for cDNA conversion and mRNA profiling while the remaining half (by volume) was processed using stem-loop primers to measure mature miRNAs. All mRNA and miRNA transcript analysis was carried out using ViiA7 real-time PCR platform using mRNA- and miRNA-specific primers (Table 1). Data were analyzed using Applied Biosystems ViiA7 Software. The results were normalized to reference mRNA (18s rRNA) or miRNA (U6) transcripts and presented as fold over detectable (FoD). 18s rRNA and U6 expression were confirmed to be uniform across all single cells. All reagents for TaqMan-based real-time quantitative PCR for gene and microRNA transcripts were obtained from Thermo Fisher Scientific, Waltham, MA.

Statistics: All data were obtained from normal (non-diabetic) human islets (N=12), T2D donor islets (N=8) and smooth muscle (N=6). Correlations were determined using Spearman r (rs) value and the p-value was calculated for each correlation. Data were analyzed and plotted using GraphPad Prism 7. Statistical significance was taken at p<0.05.

Results:

Expression of glucokinase and miR-206 in human islets
We assessed expression of GCK transcripts and miRNA-206 in normal islets, smooth muscle, and T2D islets. We observed significantly higher expression of GCK in human islets as compared to muscle (Fig. 1A). However, miR-206 was present at similar levels in both tissues (Fig. 1A). High-fat diet increased miR-206 expression in mouse islets (21); similar increases were not observed in human T2D islets. In fact T2D islets continued to express similar transcript abundance for miR-206 as that from normal glucose tolerance (NGT) donors. In miR-206 KO mice gck transcripts were 13-fold higher than in wild-type control islets suggesting an inverse correlation with miR-206 (21). However, miR-206 and GCK mRNA levels were not correlated in human islets (Fig. 1A).

MiR-206 expression in single cells from islets
Gene expression data carried out on tissue samples represent population analysis, which averages the variation amongst the millions of single cells in a tissue. We, therefore, dissociated human islets into single cells and carried out single cell PCR using the techniques established in our lab(10). GCK transcripts and miRNA-206 were analyzed to address heterogeneity between intra-islet cells and to identify if miR-206 and GCK transcripts exhibit any correlation specifically in pro-insulin transcript
containing (β-) cells alone or in other single cells isolated from islets. Single cell PCR offers the ability to test these differences as such effects may get diluted out during whole islet studies. However, we did not observe any correlation in miR-206 and GCK expression at single cell resolution (Fig. 1B). Single cells expressing very high levels of insulin (β-cells)/glucagon (α-cells) also did not demonstrate any correlation for GCK and miR-206 (Fig. 1B). Our studies point to the possibility that miR-206 may not exhibit a regulatory effect on GCK in human islets or in human β-cells.

Prediction of miR-206 sites in human and mouse GCK transcript untranslated regions

In order to further test the possibility of miR-206 targeting human GCK mRNA, we initiated in silico analyses. The human GCK transcript (ENST00000403799.7) exon 10 was aligned with the homologous section of murine gck transcript (ENSMUST00000109823.8) (Fig. 1C). The region to which miR-206 binds murine gck 3’UTR is not conserved in the human GCK 3’UTR and the overall sequence of UTRs of the two species is generally not well conserved. Moreover, although miR-206 is predicted to target the murine gck 3’UTR, this is not the case for human GCK 3’UTR (www.targetscan.org) (Fig. 1D). Thus, the absence of miR-206 correlation with GCK transcript in intact human islets as well as in dissociated human islet single cells is concordant with the absence of a miR-206 site in the human GCK 3’UTR.

Discussion

Glucokinase is an important enzyme in β-cells that ensures entry of glucose into the glycolytic pathway and insulin secretion only when the circulating glucose is increased in the absorptive phase. Gck transcript regulation by miR-206 in mouse islets is potentially an important finding(21). However, the lack of similar studies in human islets does not help in understanding the future human clinical implications of these results. We observed no correlation between GCK and miR-206 transcripts in human islets (Fig. 1A) and our analyses at single-cell resolution also failed to demonstrate any correlation between miR-206 and GCK mRNA levels (Fig. 1B), suggesting that miR-206 may not have as prominent a role in the regulation of glucokinase in human islets; contradictory to the observations reported for mouse islets. The differences in mouse and human islets could be possibly explained through three potential scenarios:
i) **Sequence dissimilarities:** We note that the human GCK 3’UTR, unlike the murine 3’UTR, does not contain a predicted miR-206 target site (Fig. 1D), which corroborates with our observed absence of correlation between human islet miR-206 and GCK mRNA levels. In order to validate that the human GCK 3’UTR is not a target of miR-206 further studies, such as Ago2 immunoprecipitation or reporter-gene analysis of human 3’UTR sequences, should be performed.

ii) **Contribution of other miRNAs:** The miR-206 microRNA gene is located in the same miRNA primary transcript as miR-133b on chromosome 1 (human chr1:3,162,656-38,188,655 hg38, mouse chr1:20679010-20679082 mm10). We note that the studies of the miR-206 KO were performed using mice with a mixed 129SvEv and C57BL6 background (21, 22). Since these mice were not backcrossed to a pure inbred background strain, a significant portion of the genomic region surrounding the miR-206 locus in the KO mice would be derived from the Embryonic Stem (ES) cell line 129SvEv. Any difference in baseline expression of nearby genes between the 129SvEv and C57BL6 strains could also, in theory, be responsible for the observed phenotype in miR-206 KO mice (21).

iii) **Inherent differences between mouse and human islets:** Human and mouse islets differ with respect to their cellular architecture (1), susceptibility to β-cell injury (2), proliferation in vitro (8) and apoptosis signaling (9). It is therefore not surprising to observe species differences in the role of miR-206 as a regulator of GCK. Other confounding factors including BMI, age and diet of the islet donors could impact the outcomes in human studies, resulting in non-replicability with mouse data. There is a possibility that other miRNAs might be controlling GCK in human islets. A recent report suggested that miR-130a/miR-130b/miR-152 alter GCK expression in T2D/IGT human islets (16).

Overall, we believe that further studies in dissecting roles of different miRNAs that target the human GCK transcript might lead to better understanding of the intricate process of insulin secretion. Moreover, our analyses emphasize the challenges in dissecting functional roles of miRNAs and in extending these observations in model organisms to the human species.

**Author contributions:** MVJ performed all experiments, analyzed the data and wrote the manuscript draft. WW assisted in processing tissue samples and miRNA analysis. CLM and MRU organized samples for analysis and assisted with lab work. DM provided the muscle tissues. TL and HT isolated,
characterized and provided normal and T2D human islets for this work. LTD reviewed and analyzed the data and made critical inputs to the manuscript. AAH designed and planned the study and wrote the final draft with MVJ and LTD. All authors contributed to the manuscript and agreed on the final draft.

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References


**Figure legend:**

Figure 1: Glucokinase and miR-206 in human islets

(A) Spearman correlation analysis was carried out on miR-206 and GCK transcript expression datasets in normal/non-diabetic human islets (Green; N=12), smooth muscle (Black; N=6) and T2D islets (Orange; N=8). Data are presented as fold-over-detectable (FoD) (4) values for both GCK and miR-206. GCK FoD values are calculated after normalization to 18S rRNA and miR-206 after global normalization. Spearman r and P values for each tissue are indicated. Panel (B) represents Spearman correlation analysis on miR-206 and GCK transcript expression in single cells isolated from normal/non-diabetic human islets (N=84). Spearman r and P values of all single cell data are presented. Insulin- and glucagon-producing single cells were identified as those with a Ct-value ≤25 (Ins) and <27 (Gcg) and are color-coded as indicated. (C) Alignment of mouse and human glucokinase UTR at the predicted miR-206 binding site in the mouse gck UTR. Identical nucleotides are indicated with solid black vertical lines. The miR-206 binding to the mouse sequence is shown on top. Nucleotides participating in binding are indicated with a grey vertical line. (D) TargetScan vs. 7.1 (www.targetscan.org) was used to predict miR-206 binding sites in the mouse and human GCK UTRs. The predicted miR-206 binding site in the mouse gck UTR is indicated with an arrow.

Table 1: Assay on Demand PCR probe and primers used in the studies

TaqMan probe-primer assay IDs (Thermo Fisher Scientific, Waltham, MA, USA) for the genes and miRNAs used in this study are provided.
Table 1: Assay on Demand PCR probe and primers used in the studies

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Gene name</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucokinase (GCK)</td>
<td>Hs01564555_m1</td>
</tr>
<tr>
<td>2</td>
<td>18S</td>
<td>Hs03003631_g1</td>
</tr>
<tr>
<td>3</td>
<td>miRNA-206</td>
<td>000510_hsa-miR-206</td>
</tr>
<tr>
<td>4</td>
<td>U6</td>
<td>001973_U6 rRNA</td>
</tr>
</tbody>
</table>
A

Gck

0 1000 2000 2500
0 2000 4000 6000
miR-206

- Islets (r = -0.07356; P = 0.8214)
- T2D islets (r = -0.07319; P = 0.8872)
- Muscle (horizontal line, no correlation)

B

Gck

0 20 40 60
0 50 100 150
miR-206

- Ins* cells
- Gcg* cells
- Non-α/β cells

C

mmu-miR-206-3p
3'GGUGUGUGAAG-GAAAGU-AAGGU 5'

Mouse gck exon 10 (3' UTR) 638 5'GACCTCCAGCCCAAACA-TTCAGAGA

Human gck exon 10 (3' UTR) 696 5'GGGTCCAGCAGTTTAGAATACCCAGAGA

D

Mouse Gck ENSMUST0000109823 3'UTR

miR-206-3p

Conserved sites for miRNA families broadly conserved among vertebrates

Mouse conserved sites for miRNA families broadly conserved among vertebrates

Human GCK ENST00000403799 3'UTR

Conserved sites for miRNA families broadly conserved among vertebrates

Human conserved sites for miRNA families broadly conserved among vertebrates