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

Mugdha V Joglekar[1], Wilson Wong[1], Cody-Lee Maynard[1], Malati R. Umrani[1], David Martin[2], Thomas Loudovaris [3], Helen E. Thomas [3], Louise T. Dalgaard[4]*, Anandwardhan A. Hardikar[1*]

1. NHMRC Clinical Trials Centre, The University of Sydney, NSW, Australia

2. Department of GI surgery, Strathfield Private Hospital, NSW, Australia

3. Immunology and Diabetes lab, St. Vincent's Institute, Fitzroy, VIC, Australia

4. Department of Science and Environment, Roskilde University, Denmark

*Correspondence: Louise Torp Dalgaard or Anandwardhan A. Hardikar

Level 6, NHMRC Clinical Trials Centre,

Medical Foundation Building, The University of Sydney,

92-94 Parramatta Road, Camperdown, NSW 2050

Australia

E-mail: ltd@ruc.dk or anand.hardikar@ctc.usyd.edu.au

Web: http://www.isletbiology.info/

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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

GCK. 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 (r_s) 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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Figure legend:

Figure 1: Glucokinase and miR-206 in human islets

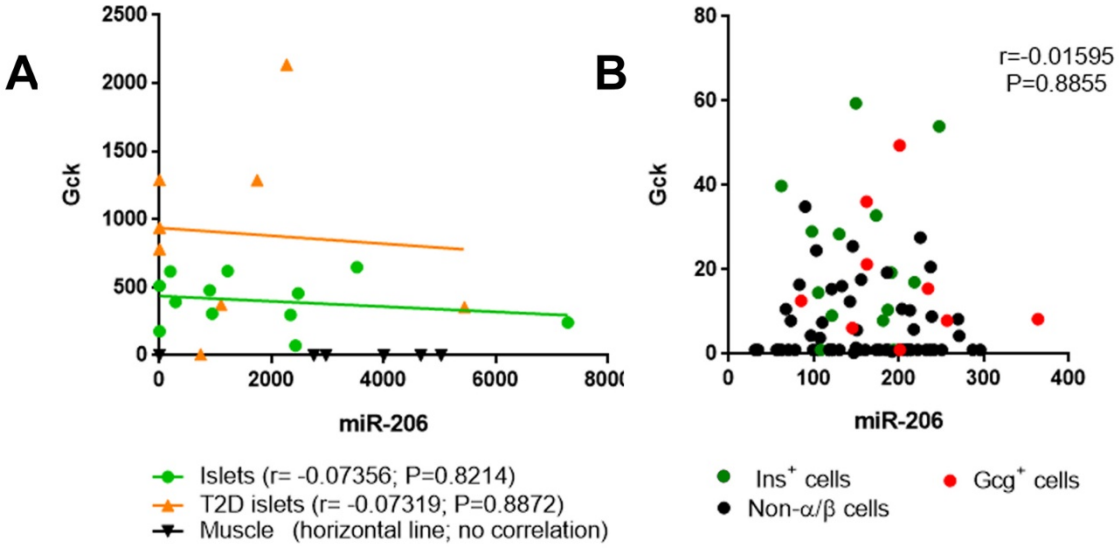
(A) Spearman correlation analysis was carried out on miR-206 and *GCK* transcript expression datasets in normal/non-diabetic human islets (Green; N=12), smooth muscle (Black; N=6) and T2D islets (Orange; N=8), Data are preseneted as fold-over-detectable (FoD) (4) values for both *GCK* and miR-206. *GCK* 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 $\leq$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

Sr. No.	**Gene name**	**Assay ID**
1	Glucokinase (GCK)	Hs01564555_m1
2	18S	Hs03003631_g1
3	miRNA-206	000510_hsa-miR-206
4	U6	001973_U6 rRNA



C

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                                    mmu-miR-206-3p
                                    3'GGUGUGUGAAG-GAAUGU-AAGGU 5'
                                       |  ||   | | ||| |||||
Mouse gck exon 10 (3'UTR) 638 5'GACTTTCCAGGCCACAAACA-TTCCAGAGA
                                       |  | ||| ||   || |  |||||||
Human gck exon 10 (3'UTR) 696 5'GGTGTCCCAAGCTTTGAATACCCCCAGAGA
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