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Genetic deletion of miR-204 improves glycemic control despite obesity in db/db mice.

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Abstract

MicroRNAs (miRs) are small non-coding RNAs that regulate the target gene expression. A change in miR profile in the pancreatic islets during diabetes is known, and multiple studies have demonstrated that miRs influence the pancreatic β -cell function. The miR-204 is highly expressed in the β -cells and reported to regulate insulin secretion. Here we investigated whether the absence of miR-204 rescues the impaired glycemic control and obesity in the genetically diabetic (*db/db*) mice. We found that the *db/db* mice overexpressed miR-204 in the islets. The *db/db* mice lacking miR-204 (*db/db-204^{-/-}*) initially develops hyperglycemia and obesity like the control (*db/db*) mice but later displayed a gradual improvement in glycemic control despite remaining obese. The *db/db-204^{-/-}* mice had a lower fasting blood glucose and higher serum insulin level compared to the *db/db* mice. A homeostatic model assessment (HOMA) suggests the improvement of β -cell function contributes to the improvement in glycemic control in *db/db-204^{-/-}* mice. Next, we examined the cellular proliferation and endoplasmic reticulum (ER) stress and found an increased frequency of proliferating cells (PCNA +ve) and a decreased CHOP expression in the islets of *db/db-204^{-/-}* mice. Next, we determined the effect of systemic miR-204 inhibition in improving glycemic control in the high-fat diet (HFD)-fed insulin-resistant mice. MiR-204 inhibition for 6 weeks improved the HFD-triggered impairment in glucose disposal. In conclusion, the absence of miR-204 improves β -cell proliferation, decreases islet ER stress, and improves glycemic control with limited change in body weight in obese mice.

Graphical abstract

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Keywords

Diabetes; β -cell function; microRNAs; endoplasmic reticulum stress; β -cell proliferation

1. Introduction:

The ever-growing incidences of diabetes constitute an enormous socioeconomic burden in the United States and across the world [1]. In 2011–2012 estimated prevalence of diabetes among U.S. adults was more than 12% [2]. MicroRNAs (miRs) are small regulatory RNAs that govern target gene expression and a change in the islets miR profile during diabetes is known [3–6]. β -cells specific disruption of the miRs network by conditional knockdown of Dicer1, an enzyme that aids in miRs synthesis, has led to the progressive development of diabetes [7]. Multiple studies demonstrate a functional influence of a single miR (e.g., miR-375 [8], miR-7 [9, 10], miR-124a [11], and miR-132 [9, 10]) on the β -cell function. MiR-204 is an abundant (in the top 10 miRs) and β -cell-selective (>50-fold over α -cells) miR [3]. Its expression is increased in the insulin-producing pancreatic tumors compared to glucagon/somatostatin producing pancreatic tumors [12]. MiR-204 targets many β -cell genes which are crucial for β -cells function and regulation of insulin synthesis/secretion [e.g., *Sirt1* & *Glp1r* [13, 14], *Dach1* [15], and *Mafa* [4, 16]]. Recent studies show elevated serum miR-204 in type 1 diabetes patients [17] and an association between serum miR-204 and cardiovascular disease risk in type 2 diabetic patients [18]. In summary, the miR-204 is abundant in the β -cells and regulates its function, however, whether it contributes to hyperglycemia and obesity *in-vivo* remains unknown. In this study, we test the hypothesis that the absence of miR-204 improves β -cell proliferation, glycemic control, and obesity during diabetes.

2. Materials and Methods:

Animals:

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Iowa and were carried out according to the National Institute of Health (NIH) guidelines. All the studies were performed in C57BL/6J, leptin-receptor mutant (*db/db*), *db/db*-miR-204 knockout (*db/db*-204^{-/-}) and miR-204^{-/-} mice of both sexes. The genotype of *db/+*, *db/db*, and *db/db*-204^{-/-} mice was confirmed by qPCR of genomic DNA from the tail snips as previously described[19]. The miR-204 inhibitor experiments were performed in the diet-induced model of insulin-resistance. Mice were given a standard normal pellet diet (NPD) or a high-fat diet (HFD). HFD is an adjusted calorie diet that provides 42% calories from fat (TD.88137, Harlan). For in vivo infusion of locked nucleic acid (LNA) microRNA inhibitor, ALZET® 2006 osmotic pumps containing oligonucleotides [scrambled control (SC) or miR-204 inhibitor (miR-204-I), Qiagen, USA] were aseptically implanted in C57BL/6J mice kept on either NPD or HFD. Mini-osmotic pumps were designed to deliver oligonucleotides at the rate of 0.15 mlh⁻¹, and each mouse received oligonucleotides at a dose of ~0.7 mgkg⁻¹day⁻¹ for 6 weeks. All mice were maintained in specific pathogen-free conditions at the central animal facility of the University of Iowa.

Determination of adiposity:

The peri-renal white adipose tissue (WAT) and interscapular brown adipose tissue (BAT) were collected and weighed. The total WAT and BAT per 100g body weight were calculated as a measure of adiposity [(WAT+BAT)/100g body weight].

Mouse islet isolation:

Pancreatic islets from mice were isolated as described previously[20]. Briefly, mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Pancreatic tissue was digested at 37°C after infusion of collagenase-P (Roche, Mannheim, Germany) to the pancreatic duct through the common bile duct clamped at its entrance to the duodenum. Next, the islets were separated from non-islets by Ficoll density gradient centrifugation. Thereafter, islets were hand-picked under a dissecting microscope and subsequently processed for RNA extraction.

Measurement of insulin and HOMA assessment.

The mice were kept on 6h fasting and the serum sample was used for measuring the fasting glucose and insulin level. The insulin levels were measured by using mouse ultrasensitive insulin ELISA Kit (ALPCO, Salem, NH). The random blood glucose level was collected from each mouse every week at 11:00 ± 1 AM. The fasting glucose and insulin data were used to generate the HOMA-IR and HOMA-β% data showing insulin-resistance and β-cells function, respectively [Formulae used are; HOMA-IR=[Fasting glucose (mmol/L) × fasting insulin (mU/L)]/22.5, HOMA-β%= 20×insulin(mU/L)/ [glucose(mmol/L)-3.5], as previously described [21, 22].

RNA isolation and qPCR:

RNA was isolated using Qiazol/Trizol as per the manufacturer's instructions. MiRs were converted to cDNA using the qscript™ microRNA cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). Real-time qPCR for miR-204 was performed using Brilliant II SYBR Green RT-qPCR kit and RNU6 was used as an internal control. Primer sequences are provided in Table 1.

Histological Processing and Immunostaining:

Sections (5 µM) of formalin-fixed paraffin-embedded pancreatic tissues were heated (95°C, 20 min) in citrate buffer (10 mM) for antigen retrieval, followed by incubation with primary antibodies. The working concentrations of anti-CHOP (Cell Signalling-2895), anti-PCNA (Cell Signalling-2586), and anti-insulin (Cell Signalling-4590) antibodies for the immunofluorescence experiments were 1:100. Antigen-primary antibody complexes were probed with fluorescence-tagged secondary antibodies. Images were captured using a Zeiss confocal microscope (Model 710). The Islet size was determined by measuring the area of insulin-stained Islets using Image J software.

Statistical analysis:

Statistical analysis was performed using GraphPad Prism (Version 8.0). One-way analysis of variance (ANOVA) was used for multiple comparisons and posthoc analysis was performed with Tukey's test. An independent sample t-test was used to determine the significance of the difference between the two groups. The significance of the difference between the two curves was analyzed by using global non-linear regression. Results were shown as mean ± s.e.m and considered significant if p values were <0.05.

3. Results:

3.1. The absence of miR-204 improves glycemic control and β-cells function.

The *db/db* mice had higher expression of miR-204 in the Islets compared to *db/+* mice (Fig. 1a). The random blood glucose level in these mice was measured once a week at 11±1 AM (without fasting). The *db/db-204^{-/-}* mice initially developed hyperglycemia similar to the *db/db* mice but later gradually demonstrated a significant improvement in glycemic control (Fig. 1b). The growth curve of *db/db-204^{-/-}* mice show that they were significantly obese compared to the *db/+* (het) mice but were slightly leaner compared to the *db/db* mice (Fig. 1c & d). We previously reported that the food and water intake of *db/db-204^{-/-}* mice was similar to that of the *db/db* mice [19]. The random blood glucose level of *db/db-204^{-/-}* mice began to divert from that of *db/db* mice beginning 10 weeks of age (Fig. 1b). Next, we measured the fasting glucose and insulin level to perform HOMA analysis as previously described [23] in 12 ± 2 weeks and 20 ± 2 weeks old mice. Although the fasting glucose level of *db/db-204^{-/-}* was better at both age points compared to the *db/db* mice, a higher serum insulin level was only observed in 20 ± 2 weeks old *db/db-204^{-/-}* mice (Fig. 1e&f). The HOMA-IR suggests both *db/db* and *db/db-204^{-/-}* mice display a similar level of impaired glucose disposal, but HOMA-β% (a measure of β-cell function) suggests that the

reversal of hyperglycemia in the *db/db*-204^{-/-} mice depends on the improvement of β -cells function (Fig. 1g&h).

3.2. Increased cell proliferation and lower endoplasmic reticulum stress in the islets of *db/db*-204^{-/-} mice.

The islets of *db/db* mice undergo architectural disorganization and enlargement[24]. Therefore, we assessed the islet enlargement and cellular proliferation in the *db/db*-204^{-/-} mice. The islets of both *db/db* and *db/db*-204^{-/-} were significantly larger than that of the *db/+* mice as expected. While the islet size did not differ between *db/db* and *db/db*-204^{-/-} mice (Fig. 2a), the *db/db*-204^{-/-} mice had a significantly higher frequency of PCNA +ve cells compared to both *db/+* and *db/db* mice (Fig. 2b & c) suggests miR-204 regulates β -cells proliferation in *db/db* mice. In support, we find that the absence of miR-204 improves β -cells ER stress as evidenced by decreased ER stress marker CHOP expression in the islets of *db/db*-204^{-/-} mice (Fig. 2d&e).

3.3. Pharmacological inhibition of miR-204 improves glucose disposal in the HFD-fed insulin-resistant mice.

To determine the potential of pharmacological intervention for miR-204 inhibition in rescuing glycemic control, the miR-204 inhibitor was systemically delivered to the HFD-fed insulin-resistant mice. MiR-204 inhibitor suppressed miR-204 expression in multiple tissues and improved glucose disposal in the glucose tolerance test in HFD-fed mice (Fig. 3a–c). However, we did not observe any improvement in the body weight, fasting blood glucose level, and adiposity in the HFD-fed mice treated with miR-204 inhibitor (Fig. 3d–f).

4. Discussion

Impaired insulin secretion and decreased β -cell mass are the key features of diabetogenesis. Intensive glycemic control using lifestyle modification, structured education, and pharmacotherapy has improved diabetes patient outcomes[25]. However, the ever-growing incidences of diabetes constitute an enormous socioeconomic burden over the U.S. and across the world[1]. There is a need to identify the newer molecular targets to improve the β -cell's function and improve metabolic control.

The islet miRs profile changes during diabetes [3–6]. As miR-204 is β -cell abundant miR [3], we investigated its role in glycemic control using *db/db* mice which spontaneously develop hyperphagia, hyperglycemia, and obesity[23]. The *db/db* mice of C57BL/6J background are resistant to islet atrophy but progressively develop hyperinsulinemia and β -cell dysfunction [26]. Our data shows a significantly better glycemic control in the *db/db*-204^{-/-} mice as evidenced by the lower fasting glucose level, random glucose level, and a higher HOMA- β %. While HOMA- β % implicates that the improvement in glucose homeostasis in miR-204 knockout *db/db* mice depends on β -cells. As all the data is generated in the global miR-204 knockout *db/db* mice it is difficult to discern the β -cells specific role of miR-204 from the secondary effect on β -cells due to the loss of miR-204 in other tissues. To ascertain the β -cell-specific miR-204's role in diabetes, additional studies

either in the β -cells-specific miR-204^{-/-} mice or targeted delivery of miR-204 inhibitor to the β -cells would be required.

The maintenance of differentiated status and proliferation preserve of β -cell mass (and proliferation) is crucial for normal glucose homeostasis. Defects in β -cell proliferation alter islet cell composition over time and can contribute to hyperglycemia and subsequently to the diabetic state. Previous studies have demonstrated that miR-204 inhibits growth, motility, migration, and invasion of cancer cells, by downregulating the CXCL8, transcription factor 12, and *ErbB3*[27–29]. The miR-204 is shown to modulates the colorectal cancer cell sensitivity in response to 5-fluorouracil-based treatment by targeting high mobility group protein A2[30]. Another study shows that miR-204 inhibits angiogenesis and promotes sensitivity to cetuximab in head and neck squamous cell carcinoma cells by blocking the JAK2-STAT3 pathway[31]. Although there is paucity on whether miR-204 regulates the proliferation of non-cancerous cells, these studies support the anti-proliferative potential of miR-204. In agreement with these studies, our results show that the absence of miR-204 increases the cell proliferation in the islets of db/db mice. Increased demand for insulin secretion primarily due to hyperglycemia and insulin resistance associated with obesity constitutes ER stress on the β -cells and is associated with β -cell apoptosis[32, 33]. A growing body of literature underlines the importance of miRs as stress regulators in β -cells during diabetes[34]. Grieco et al. reported a down-regulation of miR-204/miR-211 increased β -cells apoptosis in cytokine-treated human islets, a model mimicking type 1 diabetes [35]. In contrast, another study demonstrated that miR-204 inhibits PERK signaling and promotes β -cell ER stress and apoptosis[36]. Increased ER stress is reported in the islets affected by diabetes, and miR-204 also promotes ER stress in vascular endothelial cells[37]. We find that the absence of miR-204 decreased the expression of CHOP in the islets of db/db mice.

The *db/db* mice are widely used as a model to mimic the late stages of diabetes, but the diabetogenesis due to mutation in the leptin receptor is rare in humans with isolated cases being reported[38–40]. On the other hand, the high-fat diet (HFD) feeding mimics the early stages of diabetes, and disease pathogenesis resembles that of humans. An improvement in glucose disposal in the HFD-fed mice supports the potential of miR-204 inhibition in improving glycemic control.

The present study does not show the precise molecular mechanism through which miR-204 absence improves glycemic control apart from showing a decrease in the ER stress and an increase in cell proliferation in pancreatic islets. Comparative transcriptomics analysis of the islets of diabetic mice with or without miR-204 would help in identifying the downstream mediators of miR-204. Moreover, it would be interesting to determine whether islet miR-204 correlates with insulin secretion from the non-diabetic and diabetic islets and whether miR-204 inhibition in human islets increases insulin secretion. The increased frequency of PCNA +ve cells and a decrease in the ER stress could be an effect of miR-204 absence or secondary to better glycemic control, or a combination of both. Besides, our discovery that the gut microbiota remotely regulates miR-204 in the blood vessels of *db/db* mice[19] raises a possible influence of intestinal microbiota on the β -cell's miR-204 and the risk of diabetes.

In conclusion, miRs represent an attractive pharmacological target considering that these molecules can be precisely and efficiently targeted. Considering that miR-204 expression is increased in islets of *db/db* mice (Fig. 1a), miR-204 may contribute to decreased cell proliferation and ER stress in islets of *db/db* mice. Further, it demonstrates that miR-204 inhibition could be a viable approach in improving the β -cell function and rescuing diabetes.

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Fig. 1. The *db/db* mice lacking miR-204 have better glycemic control despite obesity.

a) Islet miR-204 in *db/db* mice. *db/+*; n=4, *db/db*; n=16. The proportion of males to the total number of mice is shown below each bar. **b)** The age-dependent change in the random blood glucose level in the *db/+*, *db/db*, and *db/db-204^{-/-}* mice. **c & d)** Growth curve (c) and representative image (d) of *db/+*, *db/db*, and *db/db-204^{-/-}* mice. The 'b' and 'c' data is collected from >30 mice with an approximately equal proportion of males and females. **e-h)** The fasting (6h) blood glucose (e), insulin (f), HOMA-IR (g), and HOMA-β% (h) in the *db/+*, *db/+204^{-/-}*, *db/db*, and *db/db-204^{-/-}* mice at indicated age-range. The proportion of males to the total number of mice is shown below each bar. ns, non-significant, *p < 0.05, **p < 0.01, ***p < 0.001 vs. indicated group. Data shown as mean and error bar represents s.e.m. F-BGL; fasting blood glucose level.

Fig. 2. The absence of miR-204 improves β -cell proliferation and decreases ER stress.

a) Islets were stained for insulin and size was measured using ImageJ software.

Representative image shows the size of islets (magnification $\times 5$, insulin; red, counterstained with DAPI; blue). *db/+*; $n=23$, *db/db*; $n=17$, *db/db-204^{-/-}*; $n=43$. n =number of islets from 3 mice. **b**) Representative images of islets stained for insulin and PCNA (magnification $\times 20$,

counterstained with DAPI; blue). Green; insulin, Red; PCNA. **c**) Quantification of PCNA

positive cells/islet in 'b'. *db/+*; $n=14$, *db/db*; $n=9$, *db/db-204^{-/-}*; $n=8$. n =number of islets from 3 mice. **d**) Representative image showing decreased CHOP expression in the islets of

db/db-204^{-/-} mice (magnification $\times 20$). **e**) Quantification of Islet CHOP intensity in 'd'. ns; not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. indicated group. Data shown as mean and error bar represents s.e.m.

Fig. 3. Systemic miR-204 inhibition rescues HFD-induced impairment in glucose disposal.

a MiR-204 expression in aorta, heart, and muscle of HFD-fed mice receiving either scrambled control (SC) or miR-204 inhibitor (miR-204-I). $n=4-6$ (male). **b** Effect of miR-204 inhibition on glucose disposal during the oral glucose tolerance test. $n=4-5$ (male). **c** The area under the curve of 'b'. **d-f** Effect of miR-204 inhibition on the fasting blood glucose level (F-BGL) (d), body weight (e), and adiposity (f). $n=4-5$ (male). ns; not significant, * $p<0.05$; ** $p<0.01$, *** $p<0.001$ vs. indicated group. Data shown as mean and error bar represents s.e.m. NPD; normal pellet diet, HFD; high-fat diet.

Table 1.

The sequence of primers, mature microRNA-204, and microRNA-inhibitor used in the study.

Mature microRNA sequence	
microRNA-204-5p	5'-UUC CCU UUG UCA UCC UAU GCC U-3'
Primer Sequence	
microRNA-204-5p	5'-CGC TTC CCT TTG TCA TCC TA-3'
RNU6	5'-GCA AAT TCG TGA AGC GTT CC-3'
Modulators	Sequence
Scrambled control	5'-ACG TCT ATA CGC CCA-3'
microRNA-204-inhibitor	5'-AGG ATG ACA AAG GGA-3'