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Role of microRNA-483 in Pancreatic β-Cells

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ROLE OF MICRORNA-483 IN PANCREATIC β-CELLS

By

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Acknowledgements

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Abstract

Insulin is an essential hormone produced by β-cells in the pancreas. The release of insulin is tightly regulated in healthy people in order to control blood sugar level in our body. However, people with Type 2 Diabetes have insufficient insulin secretion from pancreatic β-cells, leaving to high blood sugar (hyperglycemia) and β-cell failure. microRNAs (miRNAs or miR) are newly discovered small regulatory molecules and have emerged as important regulator of cell growth, differentiation, and organ function. Altered miRNA function has been implicated in the pathogenesis of a variety of human disease, including diabetes. In this report, we focus on dissecting the physiologic function of miR-483 in regulating insulin secretion and preventing β-cell failure. We discovered that miR-483 deletion in β-cells significantly decreased insulin secretion, leading to glucose intolerance when the mice were challenged with a high-fat diet. Mouse genotyping, quantitative real-time PCR, blood glucose and insulin assays, along with glucose tolerance testing have been applied in the study.

1 Introduction

1.1 Diabetes mellitus and Pancreatic Beta-Cells

Diabetes mellitus, more broadly known as diabetes, is a chronic disease associated with abnormally high levels of sugar (glucose) in the blood. The blood glucose level is tightly controlled by insulin, a hormone produced by the pancreas. Absence or insufficient production of insulin, or an inability of the body to properly use insulin causes diabetes. As stated by the World Health Organization (WHO), last updated in 2014, diabetes affects roughly 422 million people throughout the world today. One of the most important issues regarding diabetes is it's damaging effects on other body parts such as the brain, kidneys, and heart through prolonged hyperglycemic conditions and the stress these conditions cause on the body. Such potential consequences are increased risks of stroke, heart attack, and lower limb amputations. All of the above information can be seen in Figure 1 below.

There are two major types of diabetes: type 1 and type 2 diabetes. Type 1 diabetes (T1D) is generally associated with immune-mediated destruction of pancreatic β-cells (1) which are cells found in pancreatic islets that synthesize and release insulin in response to glucose in the blood. Unfortunately, there is no known cause that underlies this destruction of the β-cells. While it can be diagnosed at any age, T1D is generally diagnosed in childhood between the ages of five and seven. Type 2 diabetes (T2D) on the other hand, is more associated with overall insulin insensitivity due to insulin resistance (2). Along with insulin resistance both types of diabetes lead to an overall hyperglycemic condition of the body. As the risks increase with the length of the hyperglycemic conditions, there are a variety of methods to treat the symptoms that come with diabetes. Such methods as maintaining "normal" blood glucose levels through meticulous observation and insulin pumps are common for diabetics. However, currently diabetes is still categorized as a chronic disease with no known cure as per the WHO.

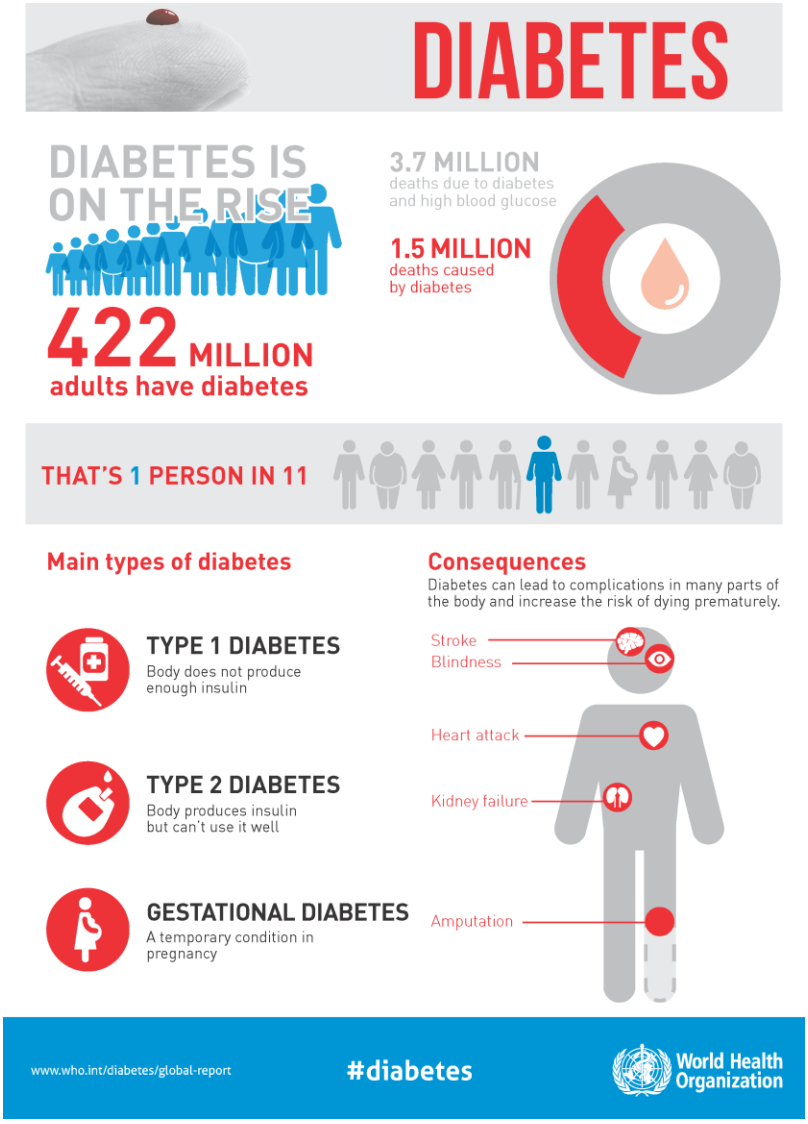

Figure 1. Classification of types of diabetes, its consequences, and its prevalence worldwide (11).

Pancreatic β-cells are the cell type that produces insulin and allows the body to maintain a regulated blood glucose level. In patients with T1D or T2D, β-cell mass and function are damaged, leading to insufficient insulin secretion such as in T1D or the non-effective use of insulin due to insulin resistance as in T2D. This, in turn, leads to hyperglycemia or hyperglycemic conditions overall (3). Due to these cells having such a large impact on diabetes they are often the target for various studies that revolve around insulin secretion and gene expression in diabetes.

1.2 Role of microRNAs in Pancreatic Beta Cells

While many transcription factors and signal proteins are most commonly involved in determining β-cell function, epigenetic regulation including microRNAs (miRNAs) introduces additional layers of temporal and spatial control that can modify β-cell function. miRNAs are small double stranded non-coding RNAs that are about 21-24 nucleotides in length (4). miRNAs act as negative regulators of gene expression by either inhibiting the translation of messenger RNAs (mRNAs) or destabilizing them. While the first miRNA was discovered long ago (1993) in C. elegans (5), many more have been discovered since then. As per miRBase, a microRNA database, there are a total of 38,589 miRNAs that have currently been discovered as of October 2018.

miRNAs work by targeting the 3'-UTR (untranslated region) in animal mRNA and the coding regions of mRNA in plants. Through their targeting of these mRNAs they are able to effectively prevent subsequent protein formation, and therefore gene expressions (6). That being said, studying them is made harder as miRNAs can target many mRNAs. Essentially the target miRNA one studies may impact other areas of the body. Currently there are many miRNAs that have been documented to relate to glucose homeostasis whether that be through insulin secretion or beta-cell mass. Some of these miRNAs can be seen in Figure 2.

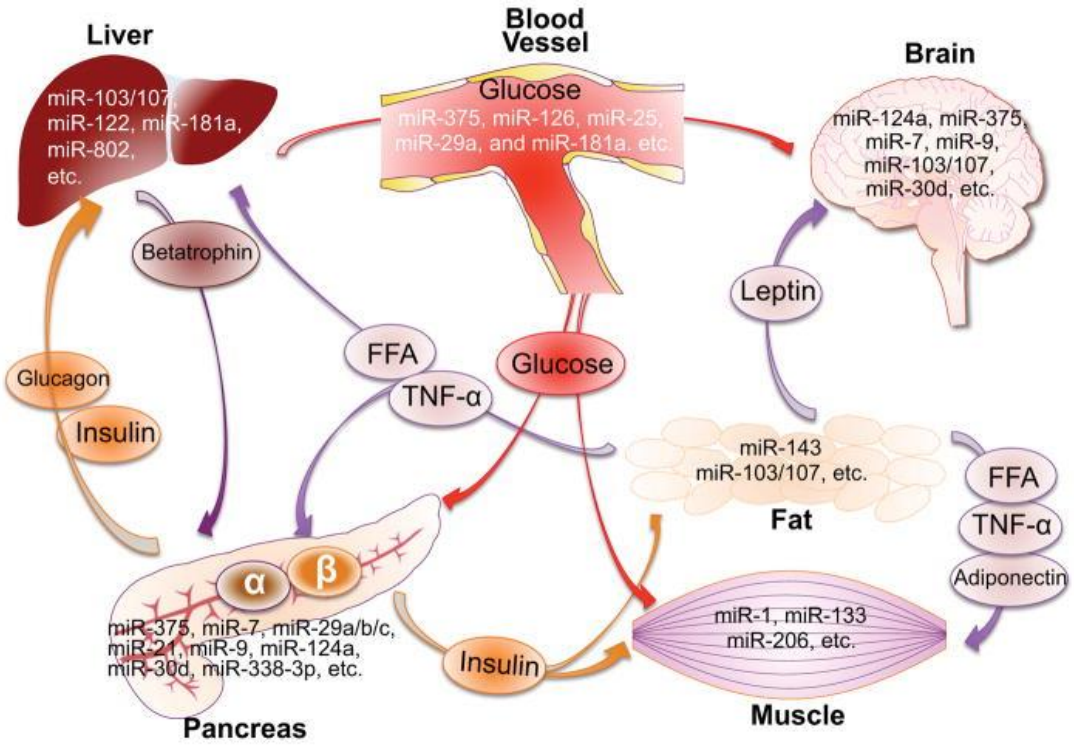

Figure 2. Crosstalk of miRNAs between tissues in glucose homeostasis regulation (9).

More recently however, there has been some headway made in the understanding of what miRNAs do and what they could be used for in regard to treatment methods. For example, one of the most important miRNAs with regards to pancreatic β-cells is miRNA-375. This miRNA is responsible for maintaining correct beta-cell mass and if beta-cells have less mass less insulin is made, resulting in a higher likelihood of diabetic symptoms (7). That being said there are plenty more that are able to regulate aspects such as insulin secretion, β-cell proliferation, and apoptosis.

A more recent miRNA that is being studied is that of miRNA-483. This miRNA is believed to have potential roles in beta cell mass and would offer another miRNA in the overall regulation of genes that impact hyperglycemic conditions. The reasoning for this is that the miRNA, more specifically miRNA-483-3p, has been observed to be highly upregulated in pancreatic cancer patients (8). Therefore, a better understanding is needed to determine the effects both upregulating and downregulating the miRNA will do, especially in cases pertaining to diabetes.

2 Materials and Methods

2.1 Animal Generation

The miR-483 floxed mice were produced at the inGenious Targeting Laboratory, Ronkonkoma, NY (www.genetargeting.com). Mice with a pancreatic beta-cell-specific deletion of miR-483 were generated by crossing miR-483 floxed mice with Ins1-Cre mice (Stock # 026801; The Jackson Laboratory) the target for knockout can be seen in Figure 3. Genotyping of all mice was performed by PCR using DNA from mouse finger biopsy. Gene specific primers are summarized in Table 1. All mice were housed in a pathogen-free animal facility, maintained on a 12-hour light/12-hour dark cycle and had free access to either regular chow diet or high-fat-diet containing 60% kCal fat (Research Diets) after weaning (4weeks). All the procedures were approved by the Animal Care Committee at Michigan Technological University.

Table 1. . PCR master mix components for mouse genotyping. Master mix 1 contained components necessary for INS-Cre mouse genotyping, while Master Mix 2 for miR-483 knockout mouse genotyping.

Master Mix 1	**Master Mix 2**
Go-taq Green: 10 µl	Go-taq Green: 10µl
Cre Forward Primer: 0.5 µl	483 Forward Primer: 1µl
Cre Reverse Primer: 0.5 µl	483 Reverse Primer: 1 µl
RNase free Water: 7 µl	RNase free water: 6 µl
Total: 18 µl	Total: 18 µl

Mater mix total = total * (Samples + NC + PC + 1). NC = negative control, PC = positive control, and add 1 with the samples to ensure enough is made for PCR.

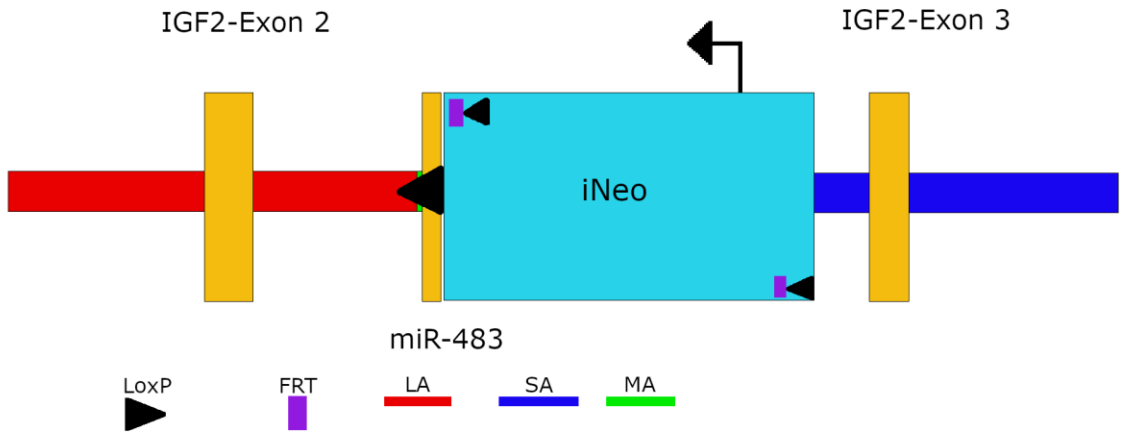

Figure 3. Structure of miR-483 floxed construct. The WT region of IGF2-miR483 gene locus is separated into three arms; long-arm (LA), middle-arm (MA), and short-arm (SA). The targeted DNA sequence contains an iNeo cassete with two sites of loxP and FRT recognition sites. These are located right after the miR-483 sequence in the second intron of the IGF2 host gene.

2.2 Islet Isolation

Pancreatic islets were isolated and purified by common bile duct perfusion of collagenase V (0.6 mg/ml) following the protocol described (10). The islets were digested in 2 mg collagenase in Hanks buffer saline for 7 minutes. The purified islets were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin for 24 to 72 hours according to the experiments.

2.3 Quantitative real-time PCR for miRNA

Total RNA from islets or cell lines was extracted using miRNeasy kit (Qiagen) according to the manufacturer's instructions and treated with rDNase I (Qiagen). TaqMan miRNA qPCR detection system (applied Biosystem) was used for quantification of miR-483. Quantitative real-time PCR (RT-qPCR) was performed using Power SYBR Green PCR Master Mix (Applied Biosystem). Real-time PCR was performed on a StepOnePlus™ System (Applied biosystem) using the following program: 10 min at 95 °C for activation of reverse DNA polymerase and 40 cycles of 95 °C for 15 sec, 60 °C for 1 min. All

samples were run in duplicate, and the RNA expression was determined using relative comparison method (ΔΔCt), with actin mRNA as an internal standard.

2.4 Body weight, Blood Glucose and Plasma Insulin Measurements

Mouse body weight was measured on a simple electronic scale. Blood glucose levels were taken by cutting off the very tip of the mouse tail and taking a blood sample. Blood glucose measurement was then taken by a glucose measurement device (ReliOn). Plasma insulin measurements were done by using the Ultrasensitive Insulin ELISA kit (Crystal Chem).

2.5 Glucose Tolerance Test

Glucose tolerance testing was carried out by fasting the mice for 16 hours prior to glucose injection. Mice were intraperitoneally (i.p.) injected with glucose in saline at 1.0 g/kg body weight. Plasma glucose levels were measured after 0, 15, 30, 45, 60, 90 and 120 min from tail vein blood.

3 Results

3.1 Confirmation of Mouse Genotypes

Mouse genotypes were confirmed using PCR and subsequent gel electrophoresis. As shown in Figure 4 the results are based upon previous samples acting as a positive control to know which bands are “lower” or “upper” bands. The distinction between having these two bands is important for future experiments as a mouse’s genotype can determine which experiments it undergoes. In addition to having a well that displays whether or not the 483 sequence is apparent, the gel also determines whether the mouse expresses Ins-Cre or not.

Figure 4. Mouse genotyping by PCR . The floxed miR-483 mice (miR483fl/fl) were crossed with Ins1-Cre mice, transgenic mice expressing Cre recombinase driven by mouse ins1 promoter to generate β-cell specific knockout mice (miR483

Once genotypes are obtained and confirmed via gel electrophoresis the mice can then be separated based upon the tests that are needed to be run. Whether they are tested on high fat diets or normal chow diets depends on the genotype of the mouse. In addition to gel electrophoresis a table of genotypes, sex, ear tag, etc. was made in order to keep mice differentiated between one another. This differentiation and data keeping can be seen in Table 2.

Table 2. An example of how data is kept in the lab to ensure data is handled well. DOB means date of birth. Ear Tags are not actual for these mice, placeholders are used instead. miR-483 -/- means the mice was a double knockout for 483, while miR-fl/fl means the mouse was floxed.

DOB: 1/17/2020	Number	Sex	Genotype	Ear Tag
	879-7	M	miR-483 -/-	75L
	879-8	F	miR-483 -/-	86R
	879-9	F	miR-483 -/-	98L
	879-10	F	mir-483 fl/fl	99L
	879-11	M	miR-483 -/-	76R
	879-12	F	miR-483 -/-	77R
	879-13	F	miR-483 -/-	84L
	879-14	F	miR-483 -/-	85R
	879-15	M	mir-483 fl/fl	96L
	879-16	M	mir-483 fl/fl	74R
	879-1/-4	M	miR-483 -/-	80L

3.2 Quantitative real-time PCR of miRNA Analysis

MicroRNA analysis was done through the use of real-time PCR methods. This allowed for an accurate representation and confirmation of miRNAs within the tissues that were being tested. Through the confirmation, real-time PCR was used to make comparisons between knockout mice and non-knockout mice. Figure 5 shows an example of results obtained from qPCR.

To validate miR-483 expression in miR-483 knockout mice, we isolated islets from miR-483 KO mice (miR-483-/-) and control mice (Ins-Cre). Compared to control mice, the expression of miR-483 was significantly decreased in isolated islets of miR-483KO mice confirmed by RT-qPCR. The results indicated that miR-483 was knock out successfully (Fig 5).

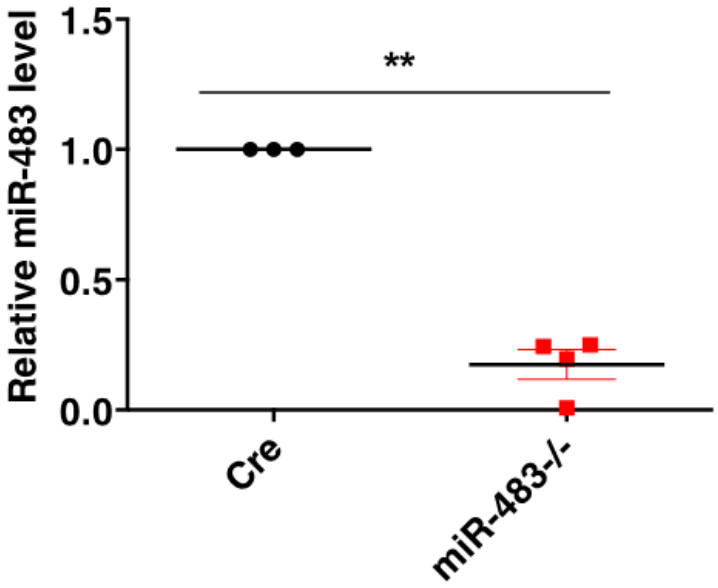

Figure 5. Confirmation of miR-483 knockout by RT-qPCR in islets isolated from miR-483-/- and Cre control mice. (** p < 0.01 vs. Cre).

3.3 Glucose Tolerance Test Analysis

Glucose tolerance testing (GTT) was done in order to complete reverse genetics and see the genotype to phenotype differences between the mice. Figure 6 shows results and subsequent analysis for the GTT and the selected mice.

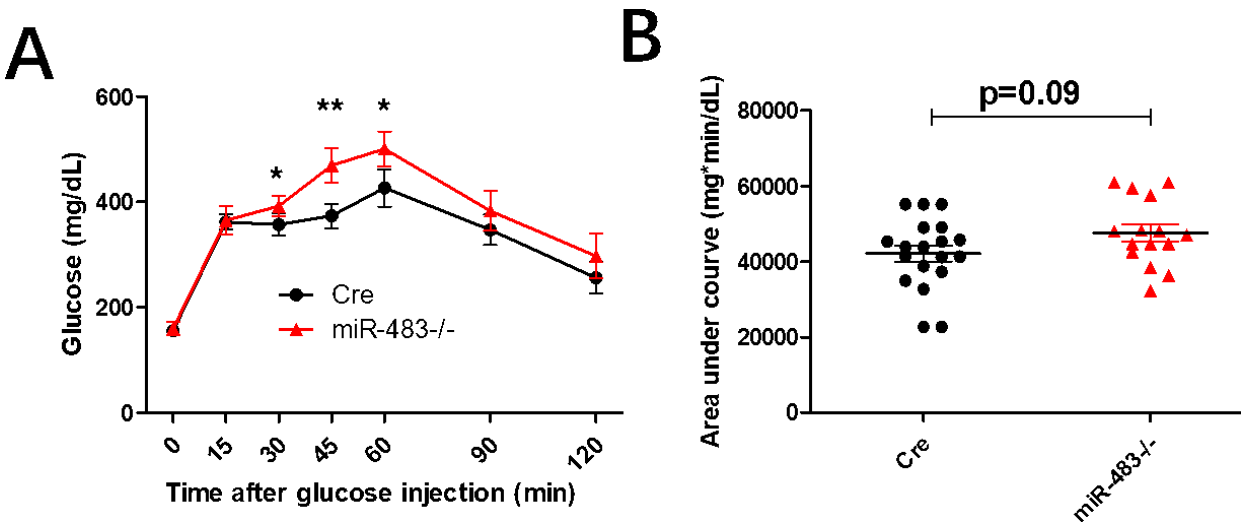

Figure 6. **A.** miR-483 deficiency increased high-fat-diet-induced glucose intolerance. **A.** Following 14 weeks of high fat diet (HFD), GTT was completed for 20-week-old male mice with genotype Cre versus miRNA-483 -/- (n=15-17 and *p<0.05 or **p<0.01). **B.** Area under the curve (AUC) calculated for the quantification of the GTT curve.

From the GTT results it was seen that the Cre, or wild type (WT) mice, had an easier time taking the glucose after injection and getting back to normal glucose levels, therefore making the WT mice have a better glucose tolerance overall. In addition, it is also shown that the double knockout mice (miR-483 -/-) had a worse glucose tolerance as the curve increases more at the 30 minutes mark before staying higher than the WT mice and lowering back down at the 120-minute mark. From this information one can say that the mice that were double knockout could have a lower tolerance for glucose, which makes them more susceptible to develop hyperglycemic conditions and subsequent diabetes. With that information future experiments for GTT and β-cell mass can be conducted in order to fill the overall picture for the effect's miRNA-483 has on pancreatic β-cells.

These results are again confirmed in the area under the curve (AUC) graph in Figure 7. Overall there is a significant difference between the Cre and miR-483 -/- mice in their overall glucose tolerance. As shown the miR-483 -/- mice had a larger AUC when compared to the Cre mice, confirming what was seen in Figure 6A. Figure 7 is provided to show the differences between insulin levels and confirm GTT results. The figure shows a significant difference between the plasma insulin levels of the mice at the 30-minute mark in the GTT test. The Cre mice have a higher level of plasma insulin meaning their pancreas is secreting insulin to deal with the glucose in the bloodstream. This means that the mice will have a higher glucose tolerance as their bodies are able to secrete insulin to deal with the glucose.

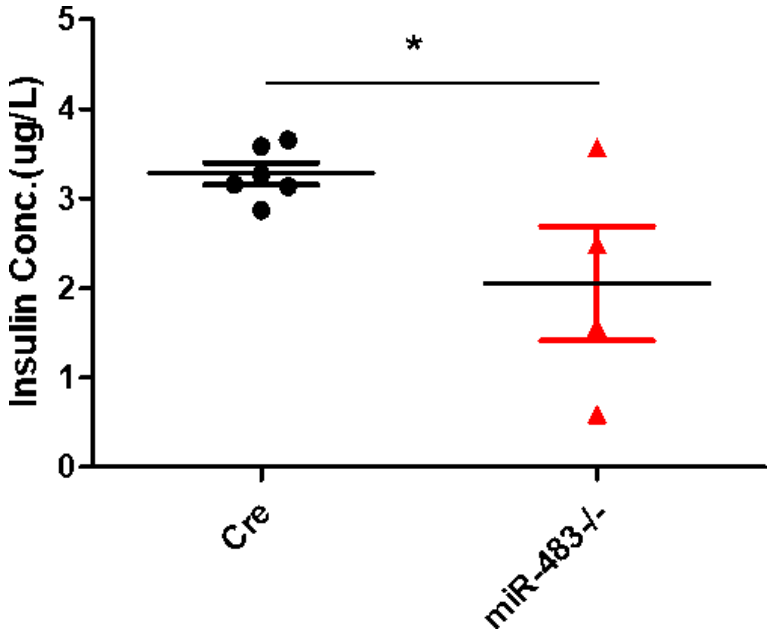

Figure 7. Plasma insulin concentration taken after 30 minutes following glucose injection (*$p<0.05$).

4 Discussion

The methods and results shown in this report go a long way to helping confirm the interactions miRNA-483 has upon pancreatic β-cells and in the prolonging of hyperglycemic conditions. While the claims cannot be set-in-stone yet, it is possible to show through GTT and qPCR that there are distinctive differences between those who have normal miRNA-483 expression and those that have less. For example, as shown in the qPCR results, we were able to confirm that the mouse was indeed a knockout (KO) for 483 due to the lower relative expression in comparison to the WT Cre mice. Although there is still some expression of miRNA-483 shown, this is due to other cells in the pancreas (such as α-cells) expressing the miRNA. With that being the case, it is possible to say that the KO mouse was successful due to the expression being so much lower.

In addition to the confirmation in qPCR, the claims can be primarily confirmed through the genotypic to phenotypic differences in glucose tolerance and insulin secretion between the mice. Mice that were knockouts for miRNA-483 had lower tolerance, or were more intolerant, to glucose through its higher AUC and lower plasma insulin when compare to the WT (Cre) mice. This data supports the claim that miRNA-483 is involved in insulin secretion and overall insulin resistance in the mouse, which can lead to hyperglycemic conditions in the mice.

Possibly the most important factor in figuring out what genotype leads to what phenotype is the genotyping of the mice. Without proper genotyping methods and ways to confirm the genotypes of the mice, one would be unable to do any subsequent analysis or experiments as the genotypes could be incorrect. In addition, without proper genotyping methods there would be many inconsistencies in finalized data, therefore it was good to be able to confirm the genotypes of the mice during many occasions. In the case for these experiments the genotyping was confirmed through multiple PCR and gel electrophoresis as needed. This meant that if there were any issues in PCR, DNA isolation, or gel electrophoresis the experiments would need to be run again and again in order to confirm validity. This occurred a few times over the course of the experiments and figuring out what the issue was always aided in present and future genotyping experiments.

For future experiments it would be good to confirm and finalize claims made surrounding miRNA-483 and its effects on pancreatic β-cells. Through more glucose tolerance testing of mice at varying ages and diets we can confirm the results obtained and say that the miRNA impacts insulin secretion and glucose tolerance in mice. From this we can then say that something similar in humans is possible and can look for ways to not only study the miRNA in humans but make treatment and diagnosis methods more available. In addition, it would also be good to see how the miRNA impacts other tissues in the body for a more generalized understanding in the case that they can be used as biomarkers for diagnosis or for further treatment methods.

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