RESEARCH

miRNA-mRNA profile and regulatory network in stearic acid-treated β -cell dysfunction

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Abstract

Chronic exposure of pancreatic β -cells to saturated fatty acid (palmitic or stearic acid) is a leading cause of impaired insulin secretion. However, the molecular mechanisms underlying stearic-acid-induced β -cell dysfunction remain poorly understood. Emerging evidence indicates that miRNAs are involved in various biological functions. The aim of this study was to explore the differential expression of miRNAs and mRNAs, specifically in stearic-acid-treated- relative to palmitic-acid-treated β -cells, and to establish their co-expression networks. β -TC-6 cells were treated with stearic acid, palmitic acid or normal medium for 24 h. Differentially expressed miRNAs and mRNAs were identified by high-throughput sequencing and bioinformatic analysis. Co-expression network, gene ontology (GO) and pathway analyses were then conducted. Changes in the expression of selected miRNAs and mRNAs were verified in β -TC-6 cells and mouse islets. Sequencing analysis detected 656 known and 1729 novel miRNAs. miRNA-mRNA network and Venn-diagram analysis yielded two differentially expressed miRNAs and 63 mRNAs exclusively in the stearic-acid group. miR-374c-5p was up-regulated by a 1.801 log₂(fold-change) and miR-297b-5p was down-regulated by a -4.669 log₂(fold-change). We found that miR-297b-5p and miR-374c-5p were involved in stearic-acid-induced lipotoxicity to β -TC-6 cells. Moreover, the effects of miR-297b-5p and miR-374c-5p on the alterations of candidate mRNAs expressions were verified. This study indicates that expression changes of specific miRNAs and mRNAs may contribute to stearic-acid-induced β -cell dysfunction, which provides a preliminary basis for further functional and molecular mechanism studies of stearic-acid-induced β -cell dysfunction in the development of type 2 diabetes.

Key Words

- ▶ pancreatic β -cell
- ▶ microRNAs
- ▶ stearic acid
- ▶ insulin secretion

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Introduction

The worldwide prevalence of type 2 diabetes mellitus (T2DM) has grown over the past three decades and continues to do so. It is characterized by insulin resistance and progressive failure of pancreatic β -cells (Smyth & Heron 2006, Li *et al.* 2012, GBD *et al.* 2016, Chen *et al.* 2017). Chronic exposure to elevated saturated fatty acid (SFA) is a major risk factor for β -cell dysfunction that can accelerate the progression of T2DM (Welsh *et al.* 2005, Risérus *et al.*

2009, Giacca *et al.* 2011). SFA, including palmitic acid (C16:0) and stearic acid (C18:0), exerts deleterious effects on β -cells (Listenberger *et al.* 2003, Elsner *et al.* 2011). Palmitic acid has long been thought to be a major regulator of β -cell function; however, emerging evidence indicates that stearic acid also plays a critical role in β -cell dysfunction (Šrámek *et al.* 2017, Acosta-Montaño *et al.* 2019, Nemecz *et al.* 2019). We previously found that, compared with

other non-esterified fatty acids (NEFAs), only stearic acid levels were dramatically increased in the postprandial serum of T2DM patients or in high-fat diet fed mice (Chu *et al.* 2013, Lu *et al.* 2016b). Meanwhile, high levels of stearic acid have a strong destructive effect on β -cells, stronger than that of palmitic acid (Lu *et al.* 2016a,b, 2018). Although generating endoplasmic reticulum (ER) stress (Lu *et al.* 2016a), alterations in calcium homeostasis (Marafie *et al.* 2019) and mitochondria dysfunction (Gehrmann *et al.* 2010) have been reported to be involved in the stearic-acid-induced lipotoxicity to pancreatic β -cells, and its precise cellular and molecular mechanisms are complex and still need to be further elucidated.

miRNAs, a large family of small (21–25 nucleotides) non-coding RNAs found in plants, animals and some viruses, play a significant role in RNA silencing and regulation of gene expression at transcriptional and post-transcriptional levels (Ambros 2004, Bartel 2018). miRNAs regulate fundamental biological processes, such as cell proliferation, differentiation, angiogenesis and apoptosis (Flynt & Lai 2008, Bartel 2018) and are also involved in multiple diseases (Jiang *et al.* 2009). Accumulated evidence indicates that miRNAs are often dysregulated in individuals suffering from T2DM (Flynt & Lai 2008, Zhu & Leung 2015, Pordzik *et al.* 2019). Zhu *et al.* observed that miR-26a regulates β -cell function and insulin signaling in db/db T2DM mice (Song *et al.* 2018). Furthermore, miR-200 was identified to induce β -cell destruction and lethal T2DM (Belgardt *et al.* 2015). However, few studies have analyzed the expression profiles of miRNAs and mRNAs in stearic-acid-induced β -cell dysfunction, and comparison between stearic acid and palmitic acid treatment has not been reported.

In the present study, we employed Illumina HiSeq sequencing to create expression profiles for both miRNAs and mRNAs in untreated and stearic-acid- or palmitic-acid-treated pancreatic β -TC-6 cells. We selected miRNAs and mRNAs that were differentially expressed in the stearic-acid-treated group and built regulatory networks to reveal potential regulatory mechanisms controlling β -cell dysfunction induced by stearic acid. Our findings provide insight into the regulatory mechanism of non-coding RNAs in the development of T2DM.

Materials and methods

Materials

Stock solutions of stearic and palmitic acids (Sigma) supplemented with BSA (3 mmol/L fatty acid: 1.5 mmol/L

BSA) were prepared as described previously (Goldstein *et al.* 1983). The working concentration for β -TC-6 cells was 400 μ mol/L. miR-297b-5p mimic, miR-374c-5p mimic, anti-miRNA-297b-5p oligonucleotides (AMO-297b-5p), anti-miRNA-374c-5p oligonucleotides (AMO-374c-5p) and their negative controls were synthesized by Guangzhou RiboBio Co. Ltd. (Guangzhou, China).

Cell culture

β -TC-6 cells were obtained from Cell Library, Chinese Academy of Sciences (Shanghai, China) and incubated at 37.0°C in a 5% carbon dioxide incubator. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1.5 g/L NaHCO₃, 15% fetal bovine serum (FBS) (Gibco) and 50 μ g/mL streptomycin together with 50 IU/mL penicillin (Gibco). The β -TC-6 cells were treated with 400 μ mol/L stearic acid or palmitic acid for 24 h. Mouse islets were isolated after collagenase P (Cat. No. 11213873001, Roche Molecular Biochemicals) digestion of pancreas by ductal injection and purified with ficoll 400 (Cat. No. 17-0300-10, Pharmacia) as described previously (Sutton *et al.* 1986).

Cell viability measurements

Cell viability was determined by assessment of lactate dehydrogenase (LDH) release and Cell Counting Kit 8 (CCK 8) assays. For measuring LDH, culture medium was collected and analyzed using an LDH assay kit (Thermo Fisher Scientific). Cell Counting Kit 8 (C0038, Beyotime Biotechnology, Shanghai, China) was used to measure cell survival rate. Absorbance was determined using a SpectraMax M2 microplate reader (Molecular Devices, San Jose, CA, USA).

Glucose stimulated insulin secretion (GSIS) assay

β -TC-6 cells were incubated in secretion buffer (pH 7.4) for 60 min with 2.8 or 20 mmol/L glucose, separately. Secretion buffer contained NaCl 129, KCl 4.8, MgSO₄ 1.2, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 5.0, and HEPES 10 (mmol/L) supplemented with 1 mg/mL BSA. The insulin level in cell culture medium was measured using a mouse/rat insulin ELISA kit (Linco Research). After collecting the supernatant for insulin measurement, the β -TC-6 cells were lysed in intermediate RIPA Lysis Buffer (Beyotime) for later evaluation of total protein content using a BCA protein assay reagent kit (Cat. No. P0010, Beyotime).

The insulin levels were standardized by every milligram protein per hour in each well.

RNA sequencing

miRNA and mRNA sequencing using the Illumina HiSeq system (San Diego, CA, USA) was performed on three samples from each of the normal, stearic acid and palmitic acid groups by GENEWIZ (Suzhou, China). Total RNA from each sample was extracted using TRIzol reagent (Invitrogen) following the manufacturer's instructions and then quantified and qualified using an Agilent 2100 Bioanalyzer (Agilent Technologies), a NanoDrop spectrophotometer (Thermo Fisher Scientific) and 1% agarose gel electrophoresis. For library preparation, 3' and 5' SR Adaptors for Illumina were ligated to miRNAs using a specific ligation enzyme. Then, first strand cDNA was synthesized by ProtoScript II Reverse Transcriptase and amplified by PCR. Sequencing was performed using a 1 × 50 single-end (SE)/2 × 150 paired-end (PE) configuration and further image analysis and base calling were conducted by the HiSeq Control Software (HCS)+OLB+GAPipeline-1.6 (Illumina) on the HiSeq instrument according to the manufacturer's instructions (Illumina).

Quality control and differential expression analysis

To achieve high quality clean data, pass filter data in FASTQ format were processed by Cutadapt (version 1.9.1) for miRNAs and Trimmomatic (v0.30) for mRNAs. All the identified miRNAs and their expression data were acquired according to miRDeep2. The DESeq/DESeq2 Bioconductor package, a model based on the negative binomial distribution, was used to perform differential expression analysis. After adjustment by Benjamini and Hochberg's approach for controlling the false discovery rate, $P < 0.05$ was set to detect differentially expressed miRNAs and mRNAs.

Functional enrichment analyses

The underlying biological function of differentially expressed miRNAs and mRNAs was predicted by functional enrichment analysis including gene ontological and pathway analysis.

Gene ontology (GO) terms, which annotate a list of enriched genes with a significant P -value less than 0.05, were identified by GO-TermFinder. We also used scripts in the Kyoto Encyclopedia of Genes and Genomes (KEGG)

database (<http://en.wikipedia.org/wiki/KEGG>) to enrich significantly differentially expressed gene in KEGG pathways.

Gene co-expression network construction

Gene co-expression networks were constructed to reveal potential interactions among selected genes. The correlation for expression of selected miRNAs and mRNAs in cell samples was calculated by Pearson correlation coefficients (PCC). Only the miRNA-mRNA pairs with the strongest correlations ($PCC \geq 0.800$, or $PCC \leq -0.800$, $P < 0.05$) were chosen to construct co-expression networks using Cytoscape software. Each gene corresponds to a node in the network.

Animal experiments

Seven-week-old male C57BL/6 mice were obtained from Vital River Laboratories Co. (Beijing, China) and randomly divided into normal diet (control, $n=10$) and high stearic acid diet (HSD, $n=10$) groups. The mice were housed in a temperature-controlled room and 60% relative humidity with non-restricted food and water. After 16 weeks, pancreatic tissue and blood samples were obtained for biochemical analysis. All animal procedures were approved by Institutional Animal Care and Use Committee of Harbin Medical University, and animals were maintained in line with the guidelines of the Animal Experimental Center of Harbin Medical University. A well-accepted approach using lard as a fat source to mimic high stearic acid diet was employed in this study, consistent with previous studies ([van den Berg *et al.* 2010](#), [Tholstrup *et al.* 2011](#), [Lu *et al.* 2016a](#), [Guo *et al.* 2020](#)). Diet composition is displayed in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article).

Profile of serum NEFAs and lipid measurement

Fasting serum NEFAs were transformed to fatty acid methyl esters as described previously ([Chu *et al.* 2013](#)). Gas chromatography-mass spectrometry analysis was performed using a TRACE gas chromatograph with a Polaris Q mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Separation was obtained on a J&W DB-WAX capillary column (30 m length, 0.25 mm inner diameter and 0.25 μ m film thickness; Agilent J&W Scientific, Folsom, CA, USA). Heptadecanoic acid (C17:0) was used as an internal standard working solution.

Table 1 Primer sequences used for qRT-PCR in this study.

Gene		Sequences (5'-3')
miR-297b-5p	Reverse transcription	GTCGTATCCAGTGCCTGTCTGCGAGTCGGCAATTGCACTGGATACGACACATGT
	Forward	GGGGATGTATGTGTGCATGA
miR-374c-5p	Reverse	GTATCCAGTGCCTGTCTGTG
	Reverse transcription	GTCGTATCCAGTGCCTGTCTGCGAGTCGGCAATTGCACTGGATACGACCACTTA
U6	Forward	GGGGATAATACAACCTGC
	Reverse	CAGTGCGTGTCTGTGGAGT
ENSMUST0000007489	Reverse transcription	CGCTTCACGAATTTGCGTGTCTAT
	Forward	GCTTCGGCAGCACATATACTA
ENSMUST00000171621	Reverse	CGCTTCACGAATTTGCGTGTCT
	Forward	ACAAGCACAGCAGACTTCAGA
ENSMUST00000086399	Reverse	GTTCTTAGCACAGTGCACCC
	Forward	ACCTTAAACGACGAGAAGCAATGG
ENSMUST00000006956	Reverse	AGCCAGACACGTAGCCCACACG
	Forward	GTCTCCAGATGACCATTATC
ENSMUST00000050785	Reverse	CCTGAAACGTATACTGGC
	Forward	TGGTCACCGTTGTGATCCC
ENSMUST00000021620	Reverse	TGAGGTCTCTTGCATG
	Forward	GAAGTTCACGGGACATGGAG
ENSMUST00000092822	Reverse	TTCAGCACATTGGGATGTTT
	Forward	CTGTCTGTCTGCCACTCCAT
ENSMUST00000149884	Reverse	GCTGGCCAAATAAGAAGAGG
	Forward	ACAAACCGCAGAGAAACAAA
ENSMUST00000021620	Reverse	CCTCCCTCTGCCCTAGTATG
	Forward	GCAGACCCAGTCGTTGTAC
ENSMUST00000073388	Reverse	AAGCCTGTGGCACAACATC
	Forward	GGCTGAAGACAGTTGTGGAA
ENSMUST00000149884	Reverse	GGGTAAATCTTGCCCTTCA
	Forward	GAGTCACTTGTCCGAGCTGTC
<i>Actb</i>	Reverse	TCGCTGTCAAGTAAAGTCCAGG
	Forward	TATTAACATAGTCAAGCCAGG
	Reverse	CACACACATATGATGCAGAT
	Forward	GGTCAGAAGGACTCCTATGTGG
	Reverse	TGTCGTCCCAGTTGGTAACA

Serum total cholesterol (TC), triacylglycerol (TG), HDL, low density lipoprotein cholesterol (LDL-C) and glucose in the fasting state were calculated with an automatic analyzer (HITACHI-7100). All kits were purchased from Biosino Biotechnology Co. Ltd. (Beijing, China).

Quantitative real-time PCR (qRT-PCR)

To confirm high-throughput sequencing results, we performed quantitative real-time PCR (qRT-PCR) on cell and mouse samples. Total RNA with miRNAs was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μ g) was reverse transcribed to cDNA in a 20- μ L reaction system using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed using Power SYBR[®] Green PCR Master Mix and a 7500 FAST Real-time PCR System (Applied Biosystems). U6 and *Actb* were used as internal controls for measurement of miRNA and mRNA levels, respectively. The primer sequences are shown in Table 1.

Statistical analysis

All data are presented as mean $\pm$ S.E.M. In Supplementary Tables 7 and 8, values are expressed as the mean $\pm$ S.D. Two-tailed Student's *t*-test and one-way ANOVA were performed to compare two or three groups, respectively, using SPSS 21.0 (Beijing Stats Data Mining Co., Beijing, China). A value of $P < 0.05$ was considered statistically significant.

Results

Stearic acid stimulates stronger lipotoxicity than palmitic acid in β -TC-6 cells

CCK 8 and LDH assays showed that lipotoxicity was significantly enhanced in the stearic-acid-treated β -TC-6 cells compared with control and palmitic-acid-treated cells (Fig. 1A and B). Meanwhile, GSIS was lower in the stearic-acid-treated cells than in cells treated with palmitic acid (Fig. 1C).

Figure 1

Stearic acid stimulates stronger lipotoxicity in β -TC-6 cells than palmitic acid. (A and B) Effect of stearic acid and palmitic acid on cell viability determined by CCK 8 and LDH assays, respectively. (C) Comparison of impaired glucose-stimulated insulin secretion (GSIS) by stearic acid and palmitic acid. * $P < 0.05$, ** $P < 0.01$, compared to the Ctrl group; ^ $P < 0.05$ vs PA group. Ctrl, control group; PA, palmitic acid; SA, stearic acid. All representative data are shown as the mean $\pm$ S.E.M. and are from triplicate experiments.

The miRNA profile and differentially expressed miRNAs in stearic-acid-treated relative to palmitic-acid-treated β -TC-6 cells

Sequencing analysis detected 656 known and 1729 novel miRNAs. Hierarchical clustering showed distinguishable miRNA expression profiles in the three groups (Fig. 2A). Differentially expressed miRNAs were screened based on $\log_2(\text{fold-change})$ and $P\text{-value} \leq 0.05$. As shown in our previous study (Guo *et al.* 2020), after palmitic acid treatment, five transcripts were significantly up-regulated and one was down-regulated, compared with the control group. In the stearic acid group, the expression level was increased for 13 miRNAs and decreased for three. Based on the Venn diagram (Guo *et al.* 2020), two miRNAs were expressed exclusively in the stearic acid group; miR-374c-5p was up-regulated by 1.801 $\log_2(\text{fold-change})$ and miR-297b-5p was down-regulated by $-4.669 \log_2(\text{fold-change})$ (Fig. 2B). In palmitic-acid-treated β -TC-6 cells, only NovelmiRNA-639 was differentially expressed with an increase of 5.107 $\log_2(\text{fold-change})$ (Supplementary Table 2), compared both with control and stearic acid group. Meanwhile, miR-21a-5p and miR-34a-5p were significantly up-regulated both in stearic and palmitic acid group (Supplementary Table 3), compared with normal group.

B

miRNA		Log2Fold Change	P-value
mmu-miR-374c-5p	PA	0.121	0.635
	SA	1.801	2.83E-12
mmu-miR-297b-5p	PA	0.005	0.034
	SA	-4.669	2.99E-63

Figure 2

The profile and differential expression of miRNAs in stearic-acid-induced β -TC-6 cells compared with palmitic-acid-treated and control groups. (A) Hierarchical clustering of differentially expressed miRNA genes in nine samples with $\log_2(\text{TPM})$ values. The dendrogram shows the relationship between expression in the control group and in cells exposed for 24 h to stearic acid or palmitic acid. Red denotes high expression levels, whereas green depicts low expression levels. From green to red, the miRNA expression increases. C, control group; S, stearic acid group; P, palmitic acid group. (B) RNA sequencing data displays the $\log_2(\text{fold-change})$ of candidate miRNAs in stearic-acid- and palmitic-acid-treated β -TC-6 cells. PA, palmitic acid; SA, stearic acid. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0055>.

The mRNA profile and differentially expressed mRNAs in stearic-acid-treated relative to palmitic-acid-treated β -TC-6 cells

To identify potential miRNAs targets, 103,640 mRNAs were detected in nine samples. Samples were clustered into three distinct groups. Hierarchical clustering of the expression profiles of mRNAs revealed that mRNA expression profiles can largely distinguish the stearic-acid and palmitic-acid-induced lipotoxicity of β -TC-6 cells (Fig. 3A). An average of 658 mRNAs was significantly up-regulated in palmitic-acid-induced β -TC-6 cells, compared with the control group, while an average of 247 mRNAs was down-regulated (Fig. 3B). In the stearic acid group, 1333 mRNAs were up-regulated and 572 were down-regulated (Fig. 3B). Of these, 63 mRNAs were specifically expressed in stearic-acid-treated β -TC-6 cells compared with the control

Figure 3

The profile and differential expression of mRNAs in stearic-acid-stimulated β -TC-6 cells compared with control and palmitic-acid-treated cells. (A) Heatmap showing the hierarchical cluster analysis of differential mRNA expression profiles with log₁₀(FPKM+1) values in stearic-acid- and palmitic-acid-treated β -TC-6 cells vs control cells. The scale bar indicates the level of mRNA expression: blue, high level of expression; red, low level of expression. From red to blue, the mRNA expression increases. C, control group; S, stearic acid group; P, palmitic acid group. (B) Screening of differentially expressed mRNAs between two groups. Up- and down-regulated genes are colored red or blue, respectively. (C) The Venn diagram shows the number of differentially expressed mRNAs between two groups. Ctrl, control; PA, palmitic acid; SA, stearic acid. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0055>.

group; 39 were up-regulated and 24 were down-regulated (Fig. 3C and Supplementary Table 4). While in palmitic acid group, 56 mRNAs were differentially and specifically expressed (Supplementary Table 5). In addition, 546 mRNAs were differentially expressed both in stearic acid and palmitic acid group, among which the top ten up- and down-regulated differentially expressed mRNAs were shown in Supplementary Table 6.

Gene ontology and pathway analysis

To identify the potential roles of differentially expressed miRNAs and mRNAs in stearic-acid-induced β -cell dysfunction, we first performed GO analysis on the basis

of biological process, cellular component and molecular function in the GO database. After setting the cut-off as $FDR \leq 0.05$, the most significant GO functions were related to molecular function, cellular component and biological process. Moreover, we further performed pathway enrichment analysis based on the pathway information in the KEGG database to clarify potential function. As shown in Figs 4 and 5, these miRNAs and mRNAs were mainly involved in GO terms, binding, cell part, cell process and biological regulation (Fig. 4A, B, C, D, E and F) and in KEGG pathways, long-term potentiation, insulin secretion, vascular smooth muscle contraction, and type 2 diabetes (Fig. 5A, B, C, D, E and F). The mRNAs were associated with apoptosis, inflammation, antioxidant activity and insulin exocytosis pathways, which are closely associated with islet β -cell function.

Construction of a miRNA-mRNA co-expression network

In an effort to reveal the relationship between miRNAs and target mRNAs specifically expressed in stearic-acid-treated β -cells, a miRNA-mRNA network was constructed as described in the Materials and methods. The network contained two miRNAs and 96 mRNA (48 positive correlated pairs and 48 negative correlated pairs, respectively) (Fig. 6A and B). The mRNA that is most positively associated with miR-297b-5p is ENSMUST00000135138 (predicted gene 15350 (*Gm15350*)) (PCC=0.996). In contrast, the most negatively related mRNA to miR-297b-5p is ENSMUST00000022531 (large tumor suppressor 2 (*Lats2*)) (PCC=-0.995). For miR-374c-5p, ENSMUST00000135828 (zinc finger protein 783 (*Zfp783*)) is the most positively correlated gene (PPC=0.990) and ENSMUST00000185596 (SLIT-ROBO Rho GTPase activating protein 2 (*Srgap2*)) is the most reversely related gene (PCC=-0.901). Additionally, as for miRNAs and target mRNAs exclusively expressed in palmitic acid group, one miRNA and 41 mRNAs (33 positive correlations and eight negative correlations, respectively) were included in the co-expression network (Fig. 6C and D), among which ENSMUST00000031327 (chemokine (C-X-C motif) ligand 1 (*Cxcl1*)) (PCC=0.996) is the most positively correlated gene and ENSMUST00000170237 (unc-51-like kinase 4 (*Ulk4*)) (PCC=-0.977) is the most negatively related gene.

Animal model characteristics

A high-stearic-acid diet fed *in vivo* mouse model mimicking increased proportions of stearic acid was successfully established, as evidenced by significant elevation of

Figure 5

Significantly enriched KEGG pathways for miRNAs and mRNAs. KEGG pathways for predicted target genes of differentially expressed miRNAs (A, B and C) and mRNAs (D, E and F). The vertical axis represents the pathway name, the horizontal axis represents enrichment factor and the size of the point represents the number of differentially expressed miRNA target genes in the pathway, while the color of the point corresponds to the Q value range. PA, palmitic acid; SA, stearic acid. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0055>.

For miR-374c-5p, no alteration was observed in skeletal muscle (Fig. 7F).

Involvement of miR-297b-5p and miR-374c-5p in stearic-acid-induced lipotoxicity to β -TC-6 cells

We then tested whether the changes in the expression of miR-297b-5p and miR-374c-5p affect β -cell viability and function. The transfection efficiency was displayed in Fig. 8A. Transfection of miR-297b-5p mimic and AMO-297b-5p alone into β -TC-6 cells apparently up-regulated and inhibited intracellular miR-297b-5p levels, respectively. Meanwhile, overexpression of miR-297b-5p significantly reversed stearic-acid-decreased miR-297b-5p expression (Fig. 8B). Furthermore, miR-297b-5p up-regulation markedly alleviated stearic-acid-induced cell death (Fig. 8C) and impaired insulin secretion (Fig. 8D). Similar results were observed after transfection of miR-374c-5p

mimic or AMO. The expression of miR-374c-5p was significantly increased and decreased by addition of its mimic and AMO, respectively (Fig. 8E). Inhibition of miR-374c-5p remarkably reversed stearic-acid-increased miR-374c-5p expression (Fig. 8F). In addition, knockdown of miR-374c-5p significantly blocked stearic-acid-stimulated decrease of cell survival rate (Fig. 8G) and reduction of insulin secretion (Fig. 8H). However, incubation of β -TC-6 cells with miR-297b-5p mimic or AMO-374c-5p alone did not significantly alter the cell viability (Fig. 8C and D) and insulin secretion (Fig. 8G and H).

Effect of miR-297b-5p and miR-374c-5p on the expressions of candidate mRNAs in stearic-acid-treated β -TC-6 cells

After transfection of miR-297b-5p mimic into β -TC-6 cells, the stearic-acid-increased expressions of

Figure 6

The co-expression network of differentially expressed miRNAs and mRNAs. (A) The candidate miRNAs are significantly correlated (positive) with corresponding mRNAs specifically in stearic-acid-treated β -TC-6 cells, compared both with palmitic acid and control group. (B) The negative correlations between miRNAs and mRNAs exclusively differentially expressed in stearic acid group. (C) The co-expression network is composed of one miRNA and its positive related mRNAs specifically in palmitic-acid-treated β -TC-6 cells, compared both with palmitic acid and normal group. (D) The miRNA is significantly reversely correlated with mRNAs specifically differentially expressed in palmitic-acid-treated cells, compared both with stearic-acid-treated and untreated cells. Genes colored in yellow and pink represent up-regulated miRNAs or mRNAs, separately. Green and blue nodes represent down-regulated miRNAs and mRNAs, respectively. Red lines refer to positive correlation and green lines represent negative correlation. A full color version of this figure is available at <https://doi.org/10.1530/JOE-20-0055>.

ENSMUST00000074898, ENSMUST00000171621 and ENSMUST0000006956 (Fig. 9A), as well as stearic-acid-decreased ENSMUST00000092822 and ENSMUST00000149884 (Fig. 9B) were significantly reversed.

When transfected with miR-297b-5p mimic alone, the five most down-regulated mRNAs ENSMUST00000124100, ENSMUST00000092822, ENSMUST00000021620, ENSMUST00000073388 and ENSMUST00000149884

Table 2 Top five up- and down-regulated differentially expressed mRNAs specifically in stearic-acid-induced β -TC 6 cells compared both with palmitic acid and control group.

Ensemble gene ID	Gene name	Log2 fold change	P Value
ENSMUST00000074898	Haptoglobin (<i>Hp</i>)	6.940	7.854E-126
ENSMUST00000171621	TRAF-interacting protein with forkhead-associated domain (<i>Tifa</i>)	6.641	2.468E-67
ENSMUST00000086399	intercellular adhesion molecule 1 (<i>Icam1</i>)	6.190	3.981E-73
ENSMUST0000006956	serum amyloid A 3 (<i>Saa3</i>)	6.127	5.323E-112
ENSMUST00000050785	lipocalin 2 (<i>Lcn2</i>)	5.881	4.656E-53
ENSMUST00000124100	prion protein gene complex (<i>Prn</i>)	-5.858	5.451E-53
ENSMUST00000092822	breast carcinoma amplified sequence 3 (<i>Bcas3</i>)	-5.561	1.597E-40
ENSMUST00000021620	OTU domain, ubiquitin aldehyde binding 2 (<i>Otub2</i>)	-5.161	1.512E-36
ENSMUST00000073388	Arylformamidase (<i>Afmid</i>)	-4.336	1.323E-24
ENSMUST00000149884	SNAP-associated protein (<i>Snapi</i>)	-4.299	1.348E-24

Figure 7

Validation of differentially expressed miRNAs and mRNAs in β -TC-6 cells and mouse islets by qRT-PCR. (A and B) Verification of candidate miRNA (miR-297b-5p and miR-374c-5p) expression in stearic-acid-treated β -TC-6 cells and high-stearic-acid-fed mouse islets. (C and D) Quantitative real-time PCR confirmation of the top five significantly up- or down-regulated mRNAs in β -TC-6 cells treated with stearic acid and in islets from high-stearic-acid diet fed mice. (E and F) Validation of miR-297b-5p and miR-374c-5p expression in stearic-acid-treated AML-12 cells and different mouse tissues fed a high-stearic-acid diet by quantitative real-time PCR. Ctrl, control group; SA, stearic acid; HSD, high-stearic-acid diet. All data are presented as the mean $\pm$ S.E.M. of three independent cell cultures or five mice per group. * P < 0.05, ** P < 0.01 and *** P < 0.001 vs the Ctrl group.

levels were apparently increased (Fig. 9B), whereas no remarkable changes were observed in the five most up-regulated expressions of ENSMUST0000074898, ENSMUST00000171621, ENSMUST00000086399, ENSMUST0000006956 and ENSMUST0000005078 (Fig. 9A). Meanwhile, the levels of ENSMUST0000074898 and ENSMUST0000006956 were increased, whereas the ENSMUST00000124100, ENSMUST00000092822 and ENSMUST00000021620 expressions were apparently reduced after incubation of AMO-297b-5p alone in the absence of stearic acid (Fig. 9A and B).

After inhibition of miR-374c-5p in stearic-acid-treated β -TC-6 cells, the down-regulated mRNA expressions of ENSMUST00000124100, ENSMUST00000021620, ENSMUST00000073388 and ENSMUST00000149884 as well as up-regulated ENSMUST00000171621 and ENSMUST0000006956 were significantly reversed, especially for ENSMUST00000124100, ENSMUST00000149884 and ENSMUST0000006956 (Fig. 10A and B). When silencing miR-374c-5p in absence of stearic acid, the five

most down-regulated mRNAs ENSMUST00000124100, ENSMUST00000092822, ENSMUST00000021620, ENSMUST00000073388 and ENSMUST00000149884 expressions were obviously increased, among which the levels of ENSMUST00000124100 and ENSMUST00000149884 were the top two highest (Fig. 10B). However, there is no significant change in the levels of the five most up-regulated mRNAs (Fig. 10A). Additionally, overexpression of miR-374c-5p only decreased the ENSMUST00000021620 mRNA level and increased ENSMUST00000074898 and ENSMUST0000006956 expressions after transfection of miR-374c-5p mimic alone, respectively (Fig. 10A and B).

Discussion

Chronic exposure to elevated SFAs is a leading cause of β -cell dysfunction in the pathogenesis and development of T2DM, which to date has no effective preventive treatment. Recently, miRNAs have become attractive

Figure 8

The role of miR-297b-5p and miR-374c-5p in stearic-acid-induced reduction of cell viability and GSIS in β -TC-6 cells. (A) The transfection efficiency of miR-297b-5p mimic and AMO in the absence of stearic acid. (B) Verification of miR-297b-5p level in the presence of stearic acid by qRT-PCR. (C) Measurement of cell viability after transfection of AMO-297b-5p in stearic acid-treated β -TC-6 cells. (D) Effect of AMO-297b-5p on GSIS after stearic acid treatment. (E) The inhibitory rate of AMO-374c-5p and over-expression rate of miR-374c-5p mimic without stearic acid treatment. (F) Alteration of intracellular miR-374c-5p expression after miR-374c-5p mimic transfection in stearic-acid-treated β -TC-6 cells. (G) Effect AMO-374c-5p on stearic-acid-decreased cell survival rate. (H) Restoration of stearic-acid-impaired GSIS after transfection of AMO-374c-5p. Ctrl, control group; NC, negative control; SA, stearic acid; 297/374mimic, miR-297b/374c-5p mimic; 297/374AMO, anti-miRNA-297b/374c-5p oligonucleotide. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs the Ctrl group; ^ $P < 0.05$, ^^ $P < 0.01$ and ^^ $P < 0.001$ vs the SA group. $n = 3$ independent cell cultures per group, and all data are presented as the mean $\pm$ S.E.M.

candidates for manipulation because of their regulatory role in mRNA transcription and protein translation. We used HiSeq sequencing technology to profile miRNAs and mRNAs and to reveal regulatory networks in SFA-impaired β -TC-6 cells, and we compared the effects of stearic acid and palmitic acid for the first time. Although the majority of studies examining elevated levels of SFA-induced worsening of β -cell function in the development of T2DM have focused on palmitic acid (Marafie *et al.* 2019, Nemezc *et al.* 2019), accumulated evidence indicates that increased levels of stearic acid have a critical role in β -cell dysfunction (Lu *et al.* 2016a, Šrámek *et al.* 2017). In this study, we demonstrated that stearic acid has a stronger effect on β -TC-6 cells than palmitic acid, similar to results in other β -cell lines (Furstova *et al.* 2008). Nevertheless, the impact of stearic acid on β -cells and the mechanisms by which it acts are poorly understood.

Pancreatic β -cell dysfunction in response to SFAs is associated with changes in the expression of protein-coding and non-coding transcripts. Recent studies show that miRNAs can serve as important regulators of β -cell functions (Plaisance *et al.* 2014, Guay & Regazzi 2015, Özcan 2015). In a previous study (Lu *et al.* 2016a), we employed gene chip technology to examine changes in miRNA levels in islets from high-stearic-acid-fed mice. However, the miRNA profile in the present study is different from that of our previous study. First, compared

with gene chip technology, high throughput sequencing is able to identify many novel unannotated genes and more effectively detects genes with lower expression. Second, the mRNA profile was determined in the present study but not in the previous project. In this study, we used β -TC-6 cells, a mouse insulinoma cell line, as a model to determine the miRNA and mRNA network after stearic acid or palmitic acid treatment. We then selected the differentially expressed miRNAs and mRNAs, especially in the stearic-acid-treated group. We validated the levels of miR-374c-5p and miR-297b-5p in various tissues from high-stearic-acid-fed mice and in cell lines treated with stearic acid. Similar changes in expression were consistently observed for these two miRNAs in primary mouse islets and β -TC-6 cells. Meanwhile, the two miRNAs were identified to be involved in stearic-acid-impaired cell viability and insulin secretion in β -TC-6 cells, of which the effect of miR-297b-5p on stearic-acid-induced reduction of cell viability and GSIS is consistent with our previous study (Guo *et al.* 2020). These findings indicate that miR-297b-5p and miR-374c-5p can potentially act as important predictors in stearic-acid-induced β -cell failure.

The second important finding of this study was to identify miRNAs and target mRNAs in β -cells that are specifically dysregulated by stearic acid and not palmitic acid. To gain insight into the potential roles of differentially expressed miRNAs in stearic-acid-induced dysfunctional

Figure 9

Alterations of candidate mRNAs expressions after transfection of miR-297b-5p mimic. (A) Effect of miR-297b-5p mimic on stearic-acid-induced five most up-regulated mRNAs levels. (B) Effect of miR-297b-5p mimic on stearic-acid-induced five most down-regulated mRNAs expressions. Ctrl, control group; SA, stearic acid; 297mimic, miR-297b-5p mimic; 297AMO, anti-miRNA-297b-5p oligonucleotide. All data are shown as the mean $\pm$ S.E.M. of three independent cell experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs the Ctrl group; ^ $P < 0.05$, ^^ $P < 0.01$ and ^^ $P < 0.001$ vs the SA group; NS, no statistical significance.

β -cells, we performed GO and pathway analysis. We found that the dysregulated miRNAs and mRNAs were most highly enriched in binding, cell part, long-term potentiation, insulin secretion, vascular smooth muscle contraction and T2DM. Besides, we selected the top five up- and down-regulated mRNAs that are differentially expressed

Figure 10

Alterations of candidate mRNAs expressions after transfection of AMO-374c-5p. (A) Changes in the expressions of five most up-regulated mRNAs after transfection of AMO-374c-5p in the presence of stearic acid. (B) The levels of top five stearic-acid-decreased mRNAs transfected with AMO-374c-5p. Ctrl, control group; SA, stearic acid; 374mimic, miR-374c-5p mimic; 374AMO, anti-miRNA-374c-5p oligonucleotide. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs the Ctrl group; ^^ $P < 0.01$ and ^^ $P < 0.001$ vs the SA group; NS, no statistical significance. Values are expressed as mean $\pm$ S.E.M. ($n = 3$).

specific to stearic acid and investigated the change in the expression of these candidate mRNAs after transfection of mimic/AMO of miR-297b-5p or miR-374c-5p into β -TC-6 cells in the presence or absence of stearic acid and then preliminarily screened the mRNAs potentially targeted by miR-297b-5p or miR-374c-5p which were involved

in the process of stearic-acid-induced β -cell dysfunction. Our results demonstrated that overexpression of miR-297b-5p could significantly reverse stearic-acid-increased ENSMUST00000074898 (*Hp*), ENSMUST00000171621 (*Tifa*) and ENSMUST0000006956 (*Saa3*) level, which indicated that miR-297b-5p may play an important role by targeting *Hp*, *Tifa* or *Saa3* in stearic-acid-stimulated lipotoxicity to β cells. For miR-374c-5p, we focused on ENSMUST00000124100 (*Prn*), ENSMUST0000021620 (*Otub2*) and ENSMUST00000149884 (*Snapin*), the expression of which was apparently inversed after inhibition of miR-374-5p with stearic acid treatment. It appears that *Prn*, *Otub2* and *Snapin* are possibly targets for miR-374c-5p in stearic-acid-induced β cell dysfunction.

Although long-term high-stearic-acid-diet-induced β -cell dysfunction has been broadly characterized as a trigger of type 2 diabetes (Lu *et al.* 2016a, Roomp *et al.* 2017), the stearic and palmitic acids, whether in the diet or serum, are actually mixed. Therefore, we further performed another critical analysis of the differentially expressed miRNAs and mRNAs profiles and their co-expression network specific to palmitic acid as well as in common to both of these two fatty acids in β -cell function. In palmitic-acid-treated cells, NovelmiRNA-639 was observed increased by 5.107 log₂(fold-change) but not detected in β -TC-6 cells exposed to stearic acid, compared with normal group. Meanwhile, miR-21a-5p and miR-34a-5p were differentially expressed both in stearic acid and palmitic acid group. These results could become a deeper knowledge of miRNA-mRNA regulatory pathway alterations in the development of SFAs induced β -cell dysfunction. This is another key finding of this study.

Alternatively, unsaturated fatty acids have been described to show protective effects in pancreatic β -cells (Morgan & Dhayal 2010, Acosta-Montaño & García-González 2018, Dhayal *et al.* 2019). In the current study, we observed that a number of unsaturated fatty acids such as linoleic acid (C18:3, LNA), palmitoleic acid (C16:1, PLA), eicosapentaenoic acid and (C20:5, EPA) were decreased in HSD mice, which would contribute to the alterations of miRNA/mRNA profiles *in vivo*.

We are well aware that the present study contains a number of limitations. First, the number of samples analyzed was relatively small. Second, we focused on the five most up- or down-regulated differentially expressed mRNAs; therefore, other mRNAs need further identification. In addition, although we found that miR-297b-5p is involved in stearic-acid-induced β -TC-6 cell apoptosis by targeting LATS2 in our previous study (Guo *et al.* 2020), the precise mechanisms and many

other direct targets by which miR-297b-5p as well as miR-374c-5p participate in stearic-acid-induced β -cell dysfunction require further exploration in *in vitro* and *in vivo* experiments.

In conclusion, we determined miRNA and mRNA expression patterns and their co-network in β -cells. Combined with bioinformatic analysis, we identified miR-374c-5p and miR-297b-5p as important nodes in the regulation of stearic-acid-induced lipotoxicity of pancreatic β -cells, especially compared with palmitic-acid-treated cells. These findings indicate an important role of miRNAs and related targets and pathways as predictors of responses to high-stearic-acid-diet-induced β -cell dysfunction in the progression of T2DM.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0055>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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H L and C S conceived and designed the experiments. Y Y, R G and Y Z performed the experiments. Y Y and H L analyzed data and wrote the manuscript. H Shi, H Sun and X W calculated the Pearson correlation coefficients. H L, Y Y and X C reviewed and edited the manuscript. The authors thank Jeremy Allen, PhD, from Liwen Bianji, Edanz Group China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

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