# Original Research

## Sigma receptor knockdown augments dysfunction and apoptosis of beta cells induced by palmitate

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### Impact statement

Most studies showed that Sigma-1 receptor (Sig-1R) is associated with nervous system disease, cancer, heart disease, retinal dysfunction, major depression, and addiction to mental stimulants. Our study linked Sig-1R to islet cells, which was previously less studied. We showed that Sig-1R silence resulted in decreased proliferation and cell cycle arrest in beta cells by regulating the FoxM1/Plk1/Cenpa pathway .What's more, Sig-1R deficiency increased beta cells sensitivity to lipotoxicity, exaggerates palmitate-induced apoptosis, and impaired insulin secretion by aggravating endoplasmic reticulum stress and mitochondrial dysfunction. This study indicated a new perspective for molecular mechanism of lipotoxicity mediating dysfunction and apoptosis of beta cells, which may provide new ideas for diabetes treatment.

### Abstract

Sigma-1 receptor (Sig-1R) is located in the endoplasmic reticulum (ER) and clustered on the mitochondria related endoplasmic membranes, which are involved in the regulation of nervous system disease. Here, we designed Sig-1R silence MIN6 cells and studied the influence of Sig-1R silence on beta cells. We showed Sig-1R inactivation in MIN6 cells could not only decrease cell proliferation but also inhibit cell cycle, and this inhibitory effect on cell cycle might be achieved by regulating the FoxM1/Plk1/Cenpa pathway.

Moreover, Sig-1R deficiency increased MIN6 cells sensitivity to lipotoxicity, exaggerated palmitate (PA)-induced apoptosis, and impaired insulin secretion. On the other hand, ER chaperone GRP78 and ER proapoptotic molecules CHOP increased in Sig-1R knockdown MIN6 cells. The ATP level decreased and reactive oxygen species (ROS) increased in this kind of cells. Furthermore not only GRP78 and CHOP levels, but also ATP and ROS levels changed more in Sig-1R silence cells after cultured with PA. Therefore, Sig-1R deficiency exaggerated PA induced beta cells apoptosis by aggravating ER stress and mitochondrial dysfunction. Together, our study showed that Sig-1R might influence the proliferation, apoptosis, and function of beta cells.

**Keywords:** Sig-1R, beta cells, lipotoxicity, apoptosis, ER stress, mitochondrial dysfunction

***Experimental Biology and Medicine*** **2021; 246: 1491–1499. DOI: 10.1177/1535370221997780**

### Introduction

The prevalence of diabetes in the world has increased dramatically from 4.6% in 2000 to 8.8% in 2015 and International Diabetic Federation predicts that the number will increase to 10.4% (642 million) by 2040.[1] Diabetes is one of the leading causes of non-communicable disease deaths now,[2] indicating that it has become a significant public health challenge worldwide. Type 1 and Type 2 diabetes are the most common types of diabetes. Beta cells apoptosis is a critical factor in the pathogenesis of both types of diabetes. Therefore, further study on the mechanism of beta cells apoptosis is vital for diabetes treatment.[3]

Mitochondria and endoplasmic reticulum (ER), as vital organelles of eukaryotic cells, are arranged in parallel ways and there are physical coupling between mitochondrial outer membranes and ER, which are called mitochondria related endoplasmic membranes (MAMs).[4] Sigma-1 receptor (Sig-1R) is located in the ER and clustered on the MAMs, which are widely distributed in the central nervous system and the peripheral system, including the lungs, liver, kidneys, pancreas, spleen, and adrenal glands.[5] Sig-1R is involved in the regulation of many diseases. So far Sig-1R has been involved with nervous system disease, cancer, heart disease, retinal dysfunction, major depression, and addiction to mental stimulants.[6] Since ER stress and oxidative stress are closely related to the impairment of beta cell function during diabetes development[7] and MAM is closely associated with hepatic insulin resistance,[8,9] muscle insulin resistance,[10] and islet cells function,[11,12] Sig-1R may also have important regulatory effects on islet cells and we investigated the influence of Sig-1R on beta cells in this study.

## Materials and methods

### Cell culture

MIN6 cells were cultured in RPMI Medium Modified (HyClone) with 10% (v/v) fetal bovine serum (Gibco), 2 mM L-glutamine, and 1% penicillin/streptomycin in humidified 5% CO2, 95% air at 37 °C.

### Creation of Sigma receptor inactivation cells

Short hairpin sequences targeting Sig-1R and the scrambled shRNA sequence were cloned into lentiviral vector GV493 (GENECHEM) labeled with GFP. The target sequences were Sig-1R-sh1,5′-GACTATTATCGCAGTGCTGAT-3′; Sig-1R-sh2,5′-CACCCTGATTCTGACTATTAT-3′; Sig-1R-sh3,5′-GAGCTTACCACCTACCTCTTT-3′.

### Real-time PCR

We used a kind of RNA extraction kit (Aidlab, China) to extract the total RNA. And then cDNA was synthesized using the reverse transcription kit named ReverTra Ace qPCR RT Kit (Toyobo, Japan). At last, real-time PCR was carried out using the Magic SYBR Mixture (Cwbio, China) in the CFX96 RT-qPCR Detection System (Bio-Rad, USA).

### Western blot analysis

First, the protein lysate was added to the treated cells to obtain the total protein. Then the protein sample was calculated according to the protein concentration. After that, the gel was prepared and put into the electrophoresis tank that was filled with electrophoresis solution. The protein marker was added for electrophoresis and then the gel was cut and transferred to polyvinylidene difluoride membranes. The membrane was dipped in the first antibody diluent overnight, and washed and soaked in the second antibody diluent for 1 h. First antibodies were as follows: anti $\beta$-actin (1: 10,000; Abcam), anti-Sig-1R (1:500; Proteintech), anti-CHOP (1: 1000; Affinity), anti-GPR78 (1: 1000; Affinity). The secondary antibodies were goat anti rabbit conjugated with horseradish peroxidase (1:10,000; Abcam). After rewashed, it was developed, exposed, and scanned in a dark room. The software Bandscan4.3 was used to analyze the gray value of the protein.

### EdU incorporation assay

We used BeyoClick™ EdU-555 kit (Beyotime, China) to measure the proliferation rate of cells. Briefly, we added EdU to the medium so that the final concentration of EdU was 20 μM and continue culturing the cells for 3 h. Then 4% paraformaldehyde was used to fix the cells and 0.3% Triton X-100 to permeabilize them. After that, we rewashed the cells using phosphate-buffered saline (PBS) with 3% bovine serum albumin (BSA) twice and then prepared Click Additive solution to incubate the cells for 30 min. Next, the cells were analyzed by flow cytometry (cytoFlex S, BECKMAN) or we continued to incubate the cells with Hoechst 33342 for 30 min and lastly observed the cells under a fluorescent microscope and took pictures of them.

### Cell cycle analysis

The two kinds of cells were digested with trypsin, washed with PBS, and fixed with ice cold 70% (vol/vol) ethanol for overnight. The next day, we washed the cells twice with PBS, added PI/RNase A(9/1,vol/vol) stain solution to incubate the cells. Lastly, we analyzed them using flow cytometry (cytoflex, BECKMAN).

### RNA sequencing

First, the total RNA was extracted and the integrity of RNA was analyzed by agarose gel electrophoresis. The mRNA with polyA tail was enriched by Oligo (dT) magnetic beads. The cDNAs were synthesized using fragments of mRNA as template and random oligonucleotides as primers. After terminal repair, adding A tails and connecting the sequencing adapter, the purified cDNA was amplified by PCR to obtain the library. Illumina sequencing was performed after the library was qualified. Sequencing fragments were transformed into sequence data by high-throughput sequencer to get raw reads and after filtering out the low-quality reads and some reads with unknown nucleotides, we could get clean reads. Clean reads were compared to the reference genome to obtain the positioning information of reads on the reference genome. According to the position information of gene alignment on the reference genome, the number of reads covered in the range from initiation to termination of each gene is counted. Next, statistical analysis was performed on the expression data to screen the genes with significantly different expression levels in different states. Then, GO function enrichment analysis and KEGG pathway enrichment analysis were performed for the differential genes.

### Cell viability assay

We use Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Japan) to measure cell viability. Cells were inoculated in 96-well plates. We added 10 μl CCK-8 solution to each hole the next day and incubate the cells for 2 h, and then the absorbance of each hole at 450 nm was determined by the enzyme labeling instrument (Bio-Tek, USA). Finally, cell survival was calculated.

### Cell apoptosis assay

Cell apoptosis rate was detected by Annexin V-APC/PI apoptosis detection kit (Key-GEN, China). We added Annexin V-APC and PI dye liquor to cell suspension according to the kit's instruction. Cells were examined by flow cytometry (cytoflex, BECKMAN) in 1 h. Annexin V-APC positive and PI negative: early apoptotic cells; Annexin V-APC positive and PI positive: late apoptotic cells. Cell apoptosis rate = (early apoptosis + late apoptosis)/total number of cells per well.

### Insulin secretion assay

When the cells were growing well, the cells were rinsed with PBS once, then the standard medium without sugar was added and incubated for 30 min. Next HEPES balanced

Krebs–Ringer bicarbonate buffer (KRBB)[13] containing 0.1% BSA solution with 2.5 mmol/L glucose was added and incubated for 1 h, and the supernatant was collected for detection of basal insulin secretion. Then 20 mmol/L glucose KRBB solution was added and incubated for 1 h, and the supernatant was collected to detect insulin secretion after glucose stimulation. Insulin concentration was measured with a mouse insulin Elisa kit (Elabscience, China).

### Reactive oxygen species assay

An appropriate amount of dihydroethidium (DHE) (Beyotime, China) powder was dissolved in PBS to prepare a DHE solution with a concentration of 5 μM. The cells were exposed to 0.5 mM palmitate (PA) for 24 h. Then the prepared DHE solution was added to incubate the cells for about 30 min. The cells were digested with trypsin, washed with PBS, and then analyzed using flow cytometry (cytoFlex S, BECKMAN).

### ATP assay

Shc and sh3 cells were cultured with or without 0.5 mM PA. At the end of incubation, the generation of ATP was measured using ATP Assay Kit (Beyotime, China). Firstly cells were lysed by lysate and the supernatant was taken after centrifugation, then the standard curve was established with ATP standard solution and the working fluid was configured.

Finally, the ATP concentration was measured on a multifunctional microporous plate detector (PE Enspire, USA).

### Statistical analysis

Results are expressed as mean ± SD. All data were analyzed with SPSS 16.0 software. Graphs were drawn with Graph-Pad Prism 8 software. Statistical significance between two experimental conditions was analyzed by using Student's t test. A $P < 0.05$ was considered statistically significant.

## Results

### Part one: Sigma receptor silence suppressed beta cells proliferation

*Creation of Sigma receptor inactivation beta cells.* We generated Sig-1R silence MIN6 cells named MIN6-sh1, MIN6-sh2, and MIN6-sh3 and created control cells (MIN6-shc) carrying a scrambled shRNA sequence. The mRNA level of Sig-1R showed reductions of Sig-1R by 30% (sh1), 48% (sh2), and 73% (sh3) compared to shc (Figure 1a). The protein level of Sig-1R showed decrease of Sig-1R by 43%, 56%, and 81% compared to shc (Figure 1b). Since sh3 target sequence was the most effective, it was used for later experiments.

*Sigma receptor silence inhibits proliferation and cell cycle of beta cells.* Sh3 cells had a lower percentage of EdU-positive cells compared with shc cells ($P < 0.05$) (Figure 2a and c) which suggested that Sig-1R silence could inhibit MIN6 cell proliferation.

Then we checked if Sig-1R silence had an influence on their cell-cycle distribution and the results showed that the percentage of G0/G1-phase cells increased and S-phase cells decreased in sh3 cells compared to shc cells ($P < 0.05$) (Figure 2b). These results indicated that the proliferation rate was lower in sh3 cells due to partial inhibition of the cycle by blockade in G0/G1 phase.

*GO and KEGG pathway analysis.* The preliminary results showed that Sig-1R silence resulted in downregulation of 663 genes so that the expression levels of these genes were

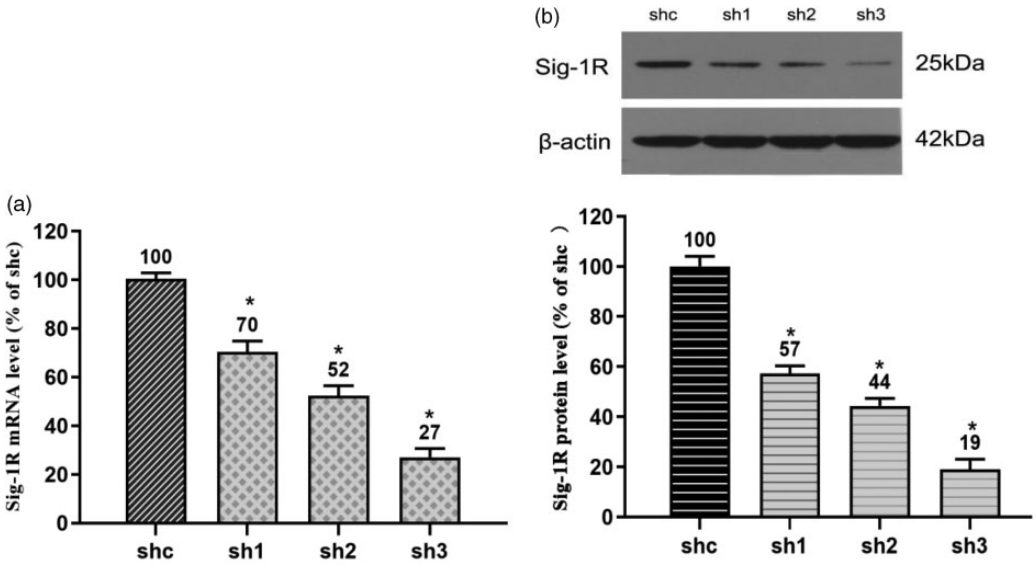


**Figure 1.** The Sig-1R expression level after Sig-1R silence: (a) Sig-1R mRNA level in cells with three different kinds of sequences targeting the Sig-1R gene. (b) The protein expression level of Sig-1R in three kinds of cells. Results are means ± SD for three observations; *$P < 0.05$. All compared with shc.

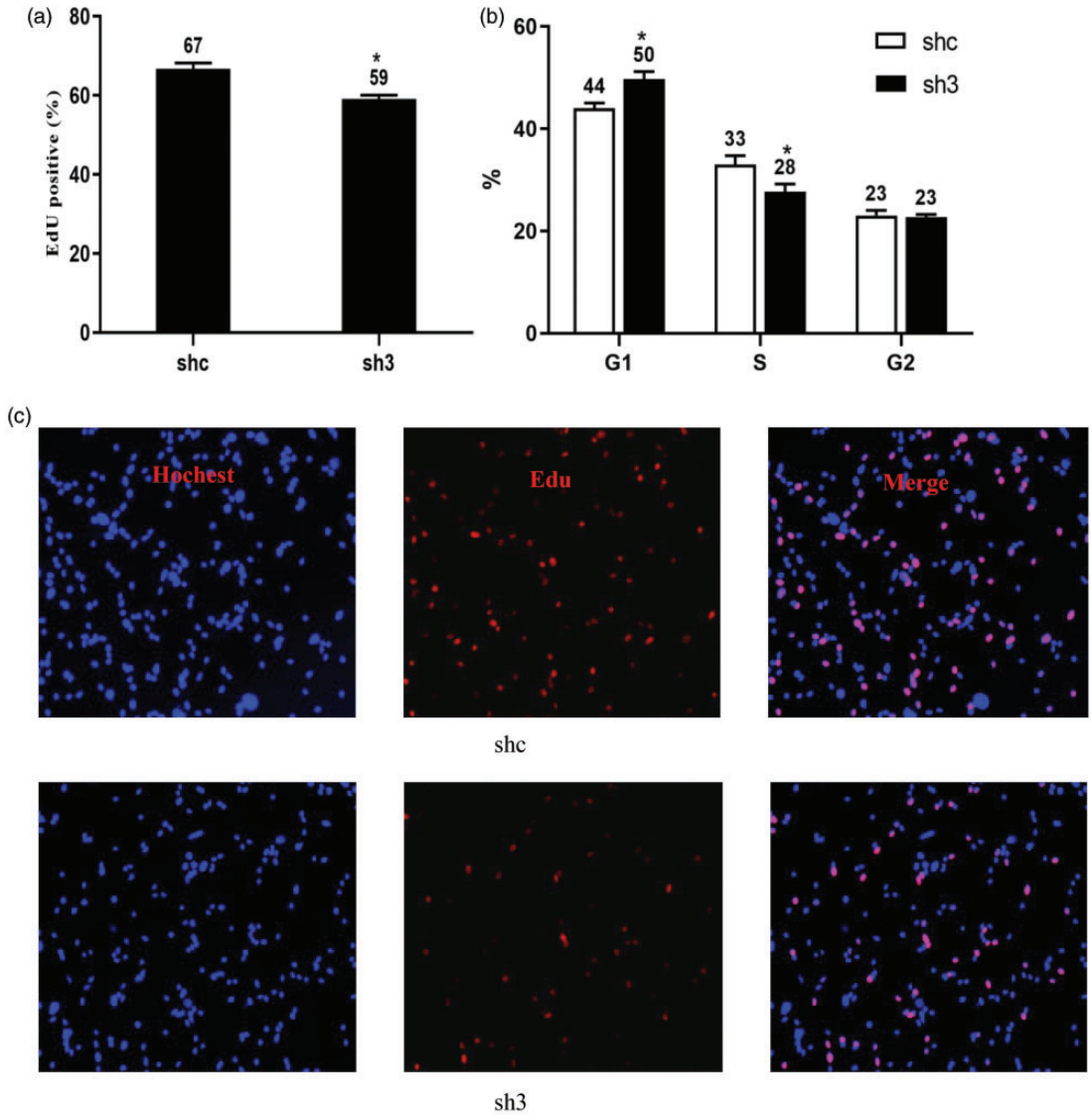


**Figure 2.** Sig-1R silence inhibited MIN6 cells proliferation: (a) Percentage of EdU positive decreased in MIN6-sh3 cells. (b) The percentage of G0/G1-phase increased and S-phase decreased in MIN6-sh3 cells. (c) Representative images (20× magnification) of MIN6 cells labeled with EdU. Results are means ± SD for three observations; *P <0.05 (compared MIN6-sh3 with MIN6-shc cells). (A color version of this figure is available in the online journal.)

all less than 83.3% compare to shc, and also resulted in 1.2-fold upregulation of 725 genes. To generate further insight view of functions of Sig-1R, GO function enrichment analysis and KEGG pathway enrichment analysis were performed. GO is a comprehensive database to describe Gene functions, which can be divided into three classes: biological process, cellular component, and molecular function. By analyzing the suppressor genes, the functions of Sig-1R were mainly involved with the following biological processes: sister chromatid segregation, mitotic sister chromatid segregation, nuclear chromosome segregation, cell division, chromosome segregation, mitotic nuclear division, DNA packaging, DNA conformation change, nuclear division, regulation of cell cycle process (Figure 3a). KEGG enriched pathways analysis showed that the top pathway of down regulated genes was cell cycle (Figure 3b). Therefore, we can conclude that knockdown of Sig-1R inhibits cell division and cell cycle.

### Part two: Sigma receptor silence has effects on apoptosis rate and insulin secretion of beta cells under lipotoxic conditions

We investigated the susceptibility of cells using CCK-8 analysis. As the result showed, the cell viability of MIN6-sh3 cells decreased more than that of shc cells after cultured with PA ($P < 0.05$) (Figure 4a) which indicated that Sig-1R silence made the cells more sensitive to lipotoxicity. We then analyzed the apoptosis rate of cells (MIN6-sh3 and shc) exposed to PA, which showed that PA greatly increased apoptotic rate of these cells and furthermore knockdown MIN6 cells increased more compared to control cells ($P < 0.05$) (Figure 4b).

Furthermore, glucose stimulated insulin secretion assays showed that insulin secretion decreased in both sh3 cells and shc cells after treated with PA, and insulin secretion decreased more in sh3 cells compared with shc cells (Figure 4c).

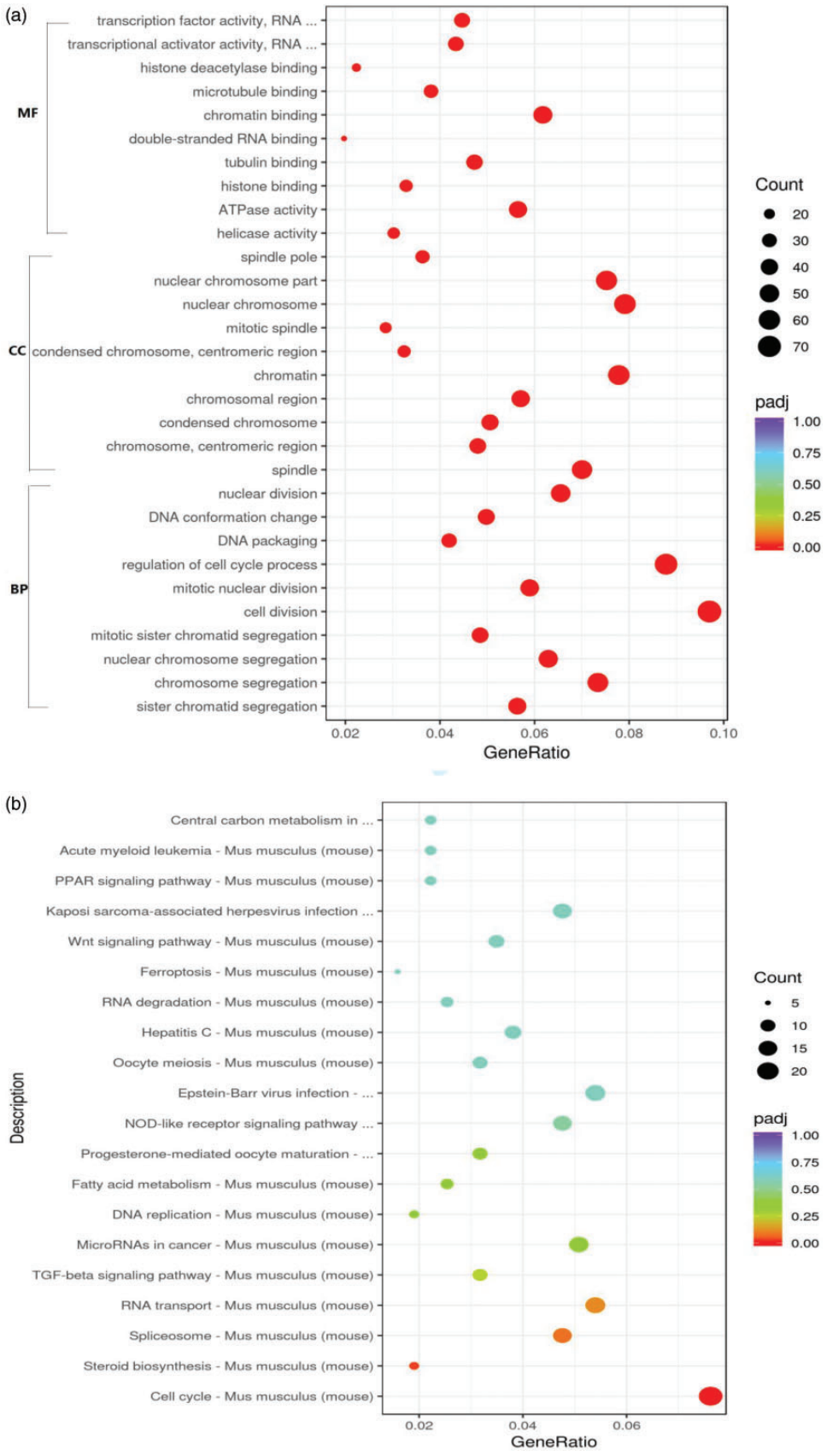


**Figure 3.** GO and KEGG pathway analysis: (a) The list of the top 10 GO terms in three classes (BP, CC, MF). (b) KEGG enriched pathways analysis showed that the top pathway of down regulated genes was cell cycle. (A color version of this figure is available in the online journal.)
BP: biological process; CC: cellular component; Count: number of differential genes in the pathway; GeneRatio: ratio of number of differential genes in this pathway to total number of differential genes; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; MF: molecular function; Padj: corrected P value for multiple hypothesis testing.

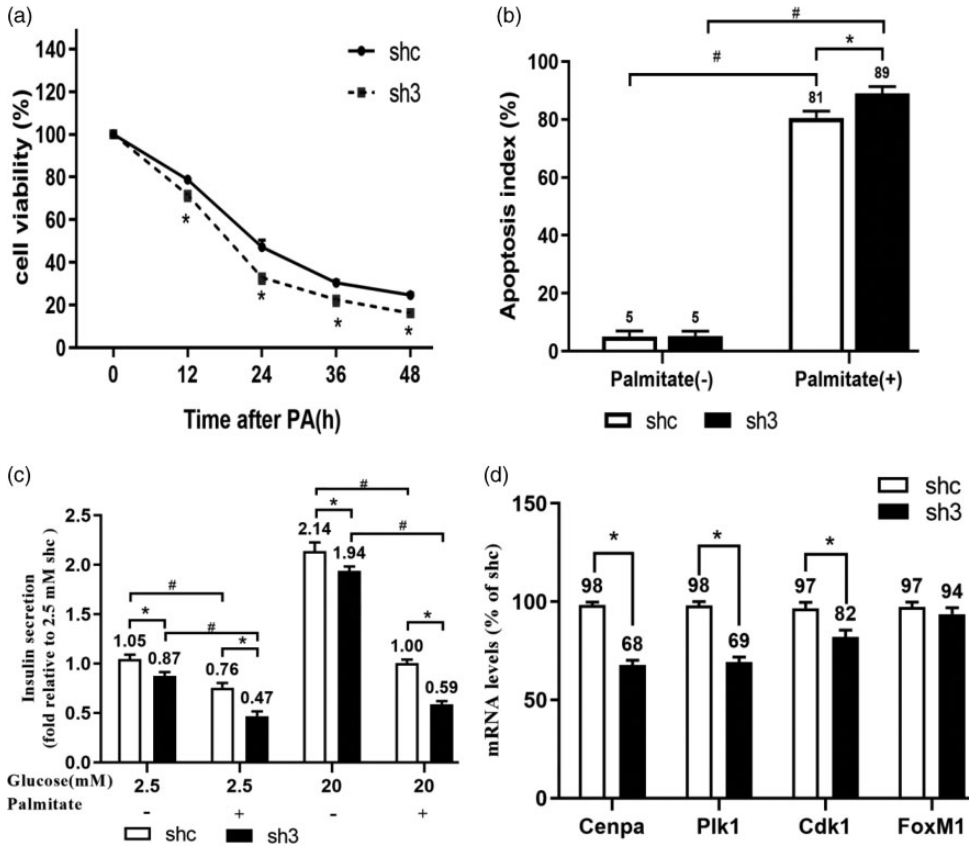


**Figure 4.** (a) Sig-1R silence increased the sensitivity of cells to palmitate: the cell viability decreased after PA was added and sh3 cells decreased more than shc. (b) Sig-1R silence enhanced apoptosis of cells induced by PA: the apoptosis rate of cells greatly increased after exposed to 0.5 mM PA for 48 h and the apoptosis rate in knockdown cells increased more compared to control cells. (c) Sig-1R silence exaggerates beta cells dysfunction induced by lipotoxicity: insulin secretion decreased in both the kinds of cells after 0.5 mM PA was added for 24 h and insulin secretion decreased more in Sig-1R silence cells. (d) Silence of Sig-1R reduced the mRNA levels of Cenpa, Plk1, Cdk1. Results are means ± SD for three observations; *P <0.05 (compared MIN6-sh3 with MIN6-shc cells); #P < 0.05 (the comparison between MIN6 cells with PA and without PA).

### Part three: The mechanism of Sigma receptor silence inhibiting cell proliferation and affecting cell function

*Sigma receptor silence suppressed the FoxM1/Plk1/Cenpa pathway in beta cells.* As mentioned above, knockdown of Sig-1R caused the downregulation of the expression of 663 genes including polo-like kinase 1 (Plk1), centromere protein A (Cenpa), cyclin-dependent kinase 1 (Cdk1). We next examined the mRNA levels of Cenpa, Plk1, Cdk1 in MIN6-shc cells and MIN6-sh3 cells. The results showed that knockdown of Sig-1R reduced their mRNA levels ($P < 0.05$) (Figure 4d), which indicated that Sig-1R silence can suppress beta cell cycle by regulating Cenpa, Plk1, Cdk1 because they are genes involved in cell cycle regulation. However, the difference in the expression of the forkhead box M1 (FoxM1) between MIN6-shc cells and MIN6-sh3 cells was not significant ($P > 0.05$).

*Sigma receptor silence aggravated ER stress and mitochondrial dysfunction induced by PA in beta cells.* The mRNA and protein levels of GRP78 and CHOP increased in sh3 cells and shc cells after cultured with PA. Furthermore, the GRP78 and CHOP expression increase more in sh3 cells ($P < 0.05$) (Figure 5a and b), which suggested that Sig-1R silence can aggravate ER stress. Furthermore, the ATP level decreased and reactive oxygen species (ROS) increased after cultured with PA in MIN6 cells. In addition, the ATP and ROS levels changed more in sh3 cells ($P < 0.05$) (Figure 5c and d). These results indicated that Sig-1R silence can aggravate ER stress and mitochondrial dysfunction induced by PA in MIN6 cells.

## Discussion

Sig-1R is a member of the Sigma receptor family which is considered to be a class of orphan receptors.[14,15] Sig-1R forms oligomers, such as trimers, thus forming a Sig-1R ligand binding bag on the ER membrane.[16] Sig-1R agonist is an effective neuroprotective agent against neurodegenerative injury,[17] which has been used to treat different neurodegenerative diseases and amyotrophic lateral sclerosis.[18] IL24 can promote tumor cell apoptosis and Sig-1R antagonists can antagonize this effect, suggesting that Sig-1R is involved in the antitumor effect of IL24.[19] In a diabetic mouse model, Sig-1R knockout mice developed retinopathy earlier than WT mice, accompanied by functional defects of retinal ganglion cells. It was found that Sig-1R

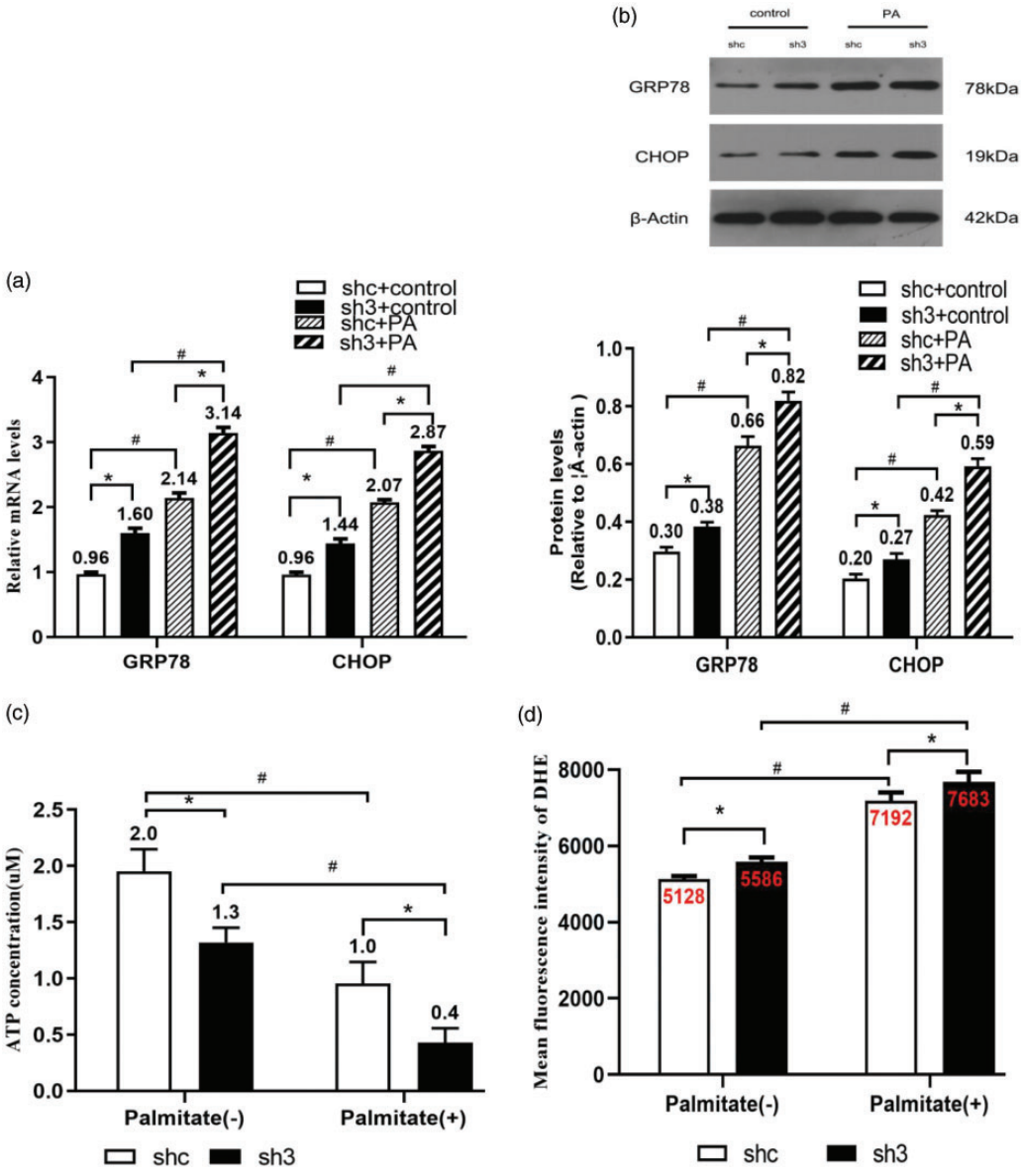


**Figure 5.** Sig-1R silence can aggravate ER stress and mitochondrial dysfunction induced by PA: (a) the mRNA expression level of GRP78 and CHOP increased after exposed to PA and increased more in knockdown MIN6 cells compared to shc cells. (b) The protein expression level of GRP78 and CHOP also increased after exposed to PA and increased more in knockdown MIN6 cells compared to shc cells. (c) The level of ATP decreased in both the kinds of cells after treated with PA and decreased more in Sig-1R silence cells. (d) The level of ROS increased in MIN6-sh3 cells and control cells after exposed to PA and increased more in knockdown MIN6 cells compared to shc cells. Results are means ± SD for three observations; $^{*}P < 0.05$ (compared MIN6-sh3 with MIN6-shc cells); $^{\#}P < 0.05$ (the comparison between MIN6 cells with PA and without PA). (A color version of this figure is available in the online journal.)

agonist lost its anti-apoptosis function after primary extraction and culture of retinal ganglion cells.[20]

To test whether Sig-1R knockdown inhibits proliferation in beta cells, we conducted experiments related to cell proliferation and cell cycle. Consistent with our hypothesis, our results suggest that Sig-1R silencing leads to reduced proliferation and G0/G1 cell cycle arrest in MIN6 cells. Furthermore, both GO and KEGG pathway analysis in our RNA sequencing showed that knockdown of Sig-1R caused cell cycle inhibition. What's more, the RNA sequencing analysis also showed downregulation of the expression of Cenpa, Plk1, Cdk1. We next examined the mRNA level of Cenpa, Plk1, Cdk1 in MIN6-shc cells and MIN6-sh3 cells and further confirmed that Sig-1R silence resulted in a reduction in these gene expressions.

Cenpa, Plk1, Cdk1 are all genes involved in cell cycle regulation. Previous reports have demonstrated that the FoxM1/Plk1/Cenpa pathway plays a vital role in beta cell proliferation.[21,22] FoxM1 can activate Cenpa and Plk1 expressions and regulate the G2/M-phase of the cell cycle.[23] Although our results didn't show significant difference in FoxM1 expression, the Cdk1expression level decreased. Cdk1 can activate FoxM1 in the nucleus but not increase its mRNA expression level.[21] Therefore, we also guess Sig-1R silence can suppress beta cell proliferation by regulating the FoxM1/Plk1/Cenpa pathway.

Previous studies have shown that lipids have toxic effects on beta cells by promoting apoptosis and reducing insulin secretion, and lipotoxicity can also damage the compensatory proliferation of beta cells.[24] Our results in this study showed that the cell viability and insulin secretion decreased and the apoptotic rate increased after PA was added to medium. What's more, the cell viability of Sig-1R knockdown MIN6 cells decreased more and the apoptotic rate of them increased more after induced by PA compared with the control cells. Then insulin secretion decreased more in Sig-1R knockdown MIN6 cells compared with the control cells. These results again confirmed the damaging effect of lipotoxicity on islet cells and also showed that Sig-1R knockdown aggravated the damaging effect. Then, we explored the mechanism.

ER and mitochondria are two most important organelles in cells. Even if they are close, their membranes will not fuse, so they can retain their unique structure and function.[25] Sig-1R, as an important chaperone molecule on MAM, has an important effect on the function of ER and mitochondria which can affect ER stress and regulate mitochondrial function.[26] Our results showed that the mRNA and protein levels of ER chaperone GRP78 and ER proapoptotic molecules CHOP increased in Sig-1R silence cells and the control cells after cultured with PA. Furthermore, the GRP78 and CHOP expressions increase more in Sig-1R silence cells. For the reason that elevated GRP78 and CHOP expressions is a marker of ER stress, we concluded that Sig-1R silence can aggravate ER stress induced by PA in MIN6 cells .Others also reported the role of Sig-1R in ER stress. Sig-1R plays a neuroprotective role by inhibiting ER stress in mouse brain. Sig-1R upregulation induced by Sig-1R ligand can alleviate neuronal damage caused by ER stress.[27,28] Ono et al.[29] found that activated Sig1R effectively inhibited the expression of GRP78 and CHOP and alleviated apoptosis in mouse hippocampal cells. Taken together, these findings suggested that Sig-1R silence might exacerbate MIN6 cell apoptosis under lipotoxic condition through aggravating ER stress.

On the other hand, Sig-1R ligands have an effect on mitochondrial respiration and ROS production,[30] which can maintain mitochondrial calcium balance and promote ATP production.[31] Our results showed that the level of ATP decreased and ROS increased in Sig-1R silence cells. And the ATP level decreased and ROS increased after cultured with PA in MIN6 cells. In addition, the ATP and ROS levels changed more in sh3 cells. Other reports also conformed the role of Sig-1R in mitochondria. Sig-1R knockdown inhibits cell growth by inducing ER stress and ROS generation in cancer cells.[32] Tagasllira et al. found that ligands activated Sig-1R to protect cardiomyocyte by increasing IP3R-mediated mitochondrial ATP production and inhibiting ER calcium loss in a stress-load-induced cardiomyocyte hypertrophy model.[33] Mitochondrial dysfunction and ER stress are closely related to islet $\beta$ cell dysfunction and participate in the development of type 2 diabetes mellitus.[34] Therefore, the decreasing insulin secretion in Sig-1R knockdown MIN6 cells might result from mitochondrial dysfunction and ER stress.

## Conclusions

In all, Sig-1R may influence MIN6 cell proliferation, insulin secretion, and cell apoptosis. However, our research needs more experiments to refine it. Firstly, we need more kinds of beta cell line and rodent models for further investigations. Furthermore, specific mechanism regulating Sig-1R particularly involving MAM needs to explore more details.

### AUTHORS' CONTRIBUTIONS

All authors contributed to the design, interpretation of the studies, and analysis of the data. The first draft of the manuscript was written by MTK. All authors read and approved the final manuscript.

### DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and publication of this article.

### FUNDING

This study was supported by the National Natural Science Foundation of China (number: 81970718).

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*(Received November 29, 2020, Accepted January 19, 2021)*