

miR-25 and miR-92b Regulate Insulin Biosynthesis and Pancreatic β -Cell Apoptosis

Zhiyi Shen

Nanjing Medical University

Yongkai Yu

Nanjing Medical University

Yuqian Yang

Nanjing Medical University

Xiao Xiao

Nanjing Medical University

Tong Sun

Nanjing Medical University

Xiaoai Chang

Nanjing Medical University

Wei Tang

Nanjing Medical University

Yunxia Zhu (✉ zhuyx@njmu.edu.cn)

Nanjing Medical University <https://orcid.org/0000-0002-4597-4445>

Xiao Han

Nanjing Medical University

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Abstract

Purpose. - Pancreatic β -cell failure is a central hallmark of the pathogenesis of diabetes mellitus; however, the molecular basis underlying chronic inflammation-caused β -cell failure remains unclear. This study reported here specifically assessed the association between miR-25/miR-92b family and β -cell failure in diabetes.

Methods. - IL-1 β and two additional ER stress activators, palmitate and tunicamycin were applied to evaluate the expression level miR-25 by Taqman[®] RT-PCR. Glucose- and potassium-stimulated insulin secretion assays were performed to assess β -cell function. Dual luciferase activity, and western blotting assays were utilized for miR-25 target gene verification. CCK-8 and TUNEL staining were used to evaluate β -cell viability and apoptosis.

Results. - miRNA ChIP identified the increased level of miR-25 in INS-1 cells by IL-1 β treatment. Expression levels of miR-25 were significantly upregulated with the treatment of IL-1 β , palmitate or tunicamycin in both INS-1 cells and human islets. Ectopic elevation of miR-25 recapitulated most featured β -cell defects caused by IL-1 β , including inhibition of insulin biosynthesis and increased β -cell apoptosis. These detrimental effects of miR-25 relied on its seed sequence recognition and repressed expression of its target genes Neurod1 and Mcl1. The miR-25/NEUROD1 axis reduced insulin biosynthesis via transcriptional regulation of β -cell specific genes. The miR-25/MCL1 axis caused β -cell apoptosis in a caspase 3/PARP1-dependent manner. Comparable impairments were generated by miR-92b and miR-25, emphasizing the redundant biological roles of miRNA family members with the same seed sequence.

Conclusion. - MiR-25/miR-92b family plays a major role in β -cell failure occurring under inflammation and diabetes states.

Introduction

Pancreatic β cells play a pivotal role in maintaining glucose homeostasis, and their ability to regulate and control insulin levels largely determines the progression of diabetes [1, 2]. The realization of glucose homeostasis requires both the recovery of β -cell numbers and the improvement of insulin synthesis and secretion. Pancreatic β -cell failure is therefore a crucial event in the pathogenesis of diabetes. The molecular mechanism that leads to islet β -cell damage, including endoplasmic reticulum stress, oxidative stress and pro-inflammatory cytokines, are being intensively investigated [3, 4].

The observation that chronic inflammation has a deleterious impact on β -cell function and survival strongly suggests a role for cytokines in β -cell deterioration. Pro-inflammatory cytokines are released by activated immune cells, including macrophages and T-cells, activating IRE1 and JUN N-terminal kinase (JNK) phosphorylation to trigger β -cell secretory dysfunction and apoptosis [1]. The regulation of β -cell failure appears to involve a complex regulatory network. However, the current theories neglect the

possible involvement of another type of gene regulator, namely microRNA (miRNA), although several studies have now indicated that miRNAs also play a critical role in β cell failure [5, 6].

The miRNAs are small, non-coding single-stranded RNA molecules that function in the post-transcriptional regulation of gene expression. These molecules mainly promote the degradation of target mRNA or inhibit translation by binding to the 3'UTR region of their target mRNAs [7, 8]. Much evidence has shown that miRNAs play an indispensable regulatory role in β -cell biology [9, 10]. In the present study, we detected global variations in miRNA expression in rat insulinoma INS-1 cells incubated with IL-1 β . Among the altered miRNAs we detected, we investigated the role of miR-25 in IL-1 β -induced β -cell damage. We demonstrated that overexpression of miR-25 suppresses insulin biosynthesis and increases β -cell apoptosis by repression of NEUROD1 and MCL1 protein expression. We further verified that miR-92b has a comparable function to that of miR-25, based on their same seed sequence. These findings reveal the mechanism of miRNA-mediated β -cell failure during the pathophysiology of diabetes and provide a potential therapeutic target for protection against diabetic β -cell failure.

Materials And Methods

Cell culture

The Mouse pancreatic β cell line MIN6 (generation 16-30) were grown in DMEM (Invitrogen, Grand Island, NY) containing 15% FBS (Gibco, Burlington, Ontario, Canada), 100 μ g/mL streptomycin, 100 units/mL penicillin, 10 mmol/L HEPES, and 50 μ mol/L β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO). The Rat pancreatic β cell line INS-1 (generation 16-30) was cultured in RPMI medium (Invitrogen) containing 10% FBS, 100 μ g/mL streptomycin, 100 units/mL penicillin, 10 mmol/L HEPES, and 50 μ mol/L β -mercaptoethanol (Sigma-Aldrich, St. Louis, MO). All cells were cultured in a humid atmosphere of 5% carbon dioxide at 37°C. The preparation method of palmitate (Sigma-Aldrich) was as described previously [11].

Primary islet isolation and culture

The research ethics committee of Nanjing Medical University approved the use of human islets. The research ethics committee of the First Affiliated Hospital of Nanjing Medical University approved the use of human islets. Isolated islets were gathered and cultured together in RPMI-1640 medium (glucose: 11.1 mmol/L) containing 10% FBS, 100 units/mL penicillin, and 100 μ g/mL streptomycin and cultured in 37°C and 5% carbon dioxide atmosphere for 3 h. For IL-1 β treatment, islets were incubated in the modified medium with 0.5% (weight for volume) BSA and IL-1 β (5 ng/ml). For palmitate or tunicamycin treatment, islets were incubated in the modified medium with 0.5% (weight for volume) BSA, various concentration of palmitate (0.25 mmol/L or 0.5 mmol/L) or tunicamycin (10 μ g/ml).

RNA extraction, quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). Then Affymetrix miRNA ChIP was applied to filtrate abundance assessment of miRNA. TaqMan[®] probes (Ambion, Foster City, CA) was used to detect miR-25 expression by using the THUNDERBIRD probe qPCR Mix (TOYOBO). For mRNA expression, SYBR Green qPCR Master Mix was used. The miRNA and mRNA expressions were detected on Roche Lightcycle480 II Sequence Detection System (Roche, Basel, Switzerland). Methods of miRNA and mRNA expression analysis were as described previously [12]. U6 and Actb were used as internal standards for miRNAs and mRNAs, respectively. Primers used are as follows:

rat MafA-F: 5'-AAGGAGGAGGTCATCCGACT-3'

rat MafA-R: 5'-TCTGGAGCTGGCACTTCTCG-3'

rat Pdx-1-F: 5'-GAAGTGCCGGCTGCCACCAT-3'

rat Pdx-1-R: 5'-CGTTCCCAGCGAGCCTGCAA-3'

rat Neurod1-F: 5'-GCCCACGCAGAAGGCAAGGT-3'

rat Neurod1-R: 5'-CCATCAGCCCGCTCTCGCTG-3'

rat Ins1-F: 5'-CATAGACCATCAGCAAGCAGG-3'

rat Ins1-R: 5'-GAAGAAACCACGTTCCCCAC-3'

rat Ins2-F: 5'-TGTCAAACAGCACCTTTGTGG-3'

rat Ins2-R: 5'-GTGCCAAGGTCTGAAGGTCAC-3'

rat Gck-F: 5'-CAGGACTTGCACTTTTACGA-3'

rat Gck-R: 5'-GCAAGGTCCAGGAAGCATT-3'

rat Glut2-F: 5'-GTCACACCAGCATAACAACACCA-3'

rat Glut2-R: 5'-ACCCACCAAAGAATGAGGCGA-3'

rat actin-F: 5'-GTCCACCCGCGAGTACAACCT-3'

rat actin-R: 5'-CGACGAGCGCAGCGATATCG-3'

Western blot analysis

INS-1 and MIN6 cells were cultured and treated as described above, and lysed with ice-cold lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 1% NP-40, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and Complete protease inhibitor (1 tablet/10 mL; Roche). After protein content determination, Western blotting was performed as previously described [13]. Individual

immunoblots were measured with antibodies to mouse anti-MCL1 monoclonal antibody, mouse anti-NEUROD1 monoclonal antibody, rabbit anti-PDX1 polyclonal antibody, rabbit anti-MAFA polyclonal antibody, rabbit anti-PARP-1 polyclonal antibody, rabbit anti-cleaved PARP-1 polyclonal antibody or rabbit anti-cleaved CASP-3 polyclonal antibody. Target protein levels were quantified relative to levels of control protein, mouse anti- α -Tubulin monoclonal antibody.

Plasmid construction

Public sources (miRanda, TargetScan, and PicTar) provided all the sequences of miR-25 response elements. The wild-type (wt) and mutant (mt) 3'UTR-luciferase constructs were generated by annealing and cloning the short sequences of rat Neurod1 and Mcl1 into pMIR-REPORT Luciferase miRNA Expression Reporter Vector (Ambion) between the SpeI and HindIII sites. To construct MCL1 expression plasmids, the coding sequence for MCL1 was amplified by PCR from the mouse full-length cDNA and then cloned into pCMV5-myc vector between the EcoR1 and Kpn1 sites. We verified the validity of plasmids by sequencing.

Transient Transfection and Luciferase Report Assay

miRNA duplex mimics and negative controls were obtained from GenePharma (Shanghai, China). For transient transfection, Lipofectamine 2000 reagent (Invitrogen) was mixed with miRNA mimics or overexpression/reporter plasmids as previously described [12]. Luciferase activities were measured using the Dual-Glo Luciferase Assay System (Promega, Madison, WI) on a TD-20/20 Luminometer (Turner BioSystems, Sunnyvale, CA) according to the manufacturer's protocols as previously described [12].

Insulin secretion assay

MIN6 cells were used for glucose-stimulated insulin secretion (GSIS) assay. INS-1 cells were used for potassium-stimulated insulin secretion (KSIS) assay. Cells were seeded in 48-well plates and treated as indicated for insulin secretion assays. After incubation for 1 h in HEPES-balanced Krebs-Ringer bicarbonate buffer (KRBH) (115 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4 \cdot 7 H_2O$, 1.2 mmol/L KH_2PO_4 , 20 mmol/L $NaHCO_3$, 16 mmol/L HEPES, 2.56 mmol/L $CaCl_2$ and 0.2% BSA), MIN6 cells were incubated for 1 h in KRBH with low glucose (2 mmol/L) and stimulatory glucose (20 mmol/L). INS-1 cells were incubated for 1 h in KRBH with low glucose (3.3 mmol/L) and KCl (50 mmol/L). After sampling, 200 μ L acid ethanol (37 ml absolute ethanol + 700 μ L concentrated hydrochloric acid, dilute with ddH₂O to 50 ml) was added per well. Then refrigerated it at 4°C for 24 h to extract insulin. The insulin levels of supernatants and extractions were measured using radioimmunoassay. Insulin radioimmunoassay kit was purchased from Beijing North Institute of Biotechnology Co., Ltd (Beijing, China). Both insulin secretion and insulin content were normalized to total protein amount.

Cell Counting Kit-8 Assay

Cells were inoculated in 48-well plates (4×10^4 cells/well) and cultured in 200 μ L culture medium. Cell Counting Kit-8 (CCK-8) assay was carried out in line with the manufacturer's protocol (Vazyme Biotech, Nanjing, Jiangsu, China).

TUNEL Staining

The In Situ Cell Apoptosis Detection Kit III (fluorescein isothiocyanate) (Boster Biological Technology, Wuhan, Hubei, China) was used in accordance with the manufacturer's protocol to carry out TUNEL Staining [14]. Observation of cells were performed under laser scanning confocal microscope (FV1200; Olympus, Tokyo, Japan).

Hoechst Staining

Hoechst 33342 staining was used to stain nuclei. Fluorescence images were obtained using Leica LAS Image Analysis software (Leica). ImageJ 1.49v software (National Institutes of Health, USA) was used to define a threshold to remove non-specific signal, and apoptotic cells were counted.

Statistical Analysis

At least three independent experiments were performed and data are expressed as mean \pm SEM. Two tailed Student's t test was applied to make comparisons between pairs of groups, or ANOVA in multiple groups. A *p* value <0.05 was considered statistically significant.

Results

Diabetes pathogenic factors increase miR-25 in human islets and INS-1 cells

Our first approach was to evaluate the change of miRNAs levels under inflammatory conditions; therefore, we treated INS-1 cells with IL-1 β for different durations and then conducted global miRNA expression profiling. We noted a significantly increased expression of miR-25 following treatment of INS-1 cells with IL-1 β (<https://www.researchsquare.com/article/rs-58542/v1;DOI:10.21203/rs.3.rs-58542/v1>; [accessed 10 September 2021]), leading us to investigate the possible involvement of miR-25 in β -cell failure.

We used a qRT-PCR assay to verify the altered expression levels of miR-25 by diabetes pathogenic factors like the pro-inflammatory cytokine IL-1 β . We found a significant increase in miR-25 expression at 12 h, and a return to the basal level at 24 h post IL-1 β treatment (Fig. 1A). We also introduced two classic ER stress activators, palmitate and tunicamycin. We observed a dose-dependent upregulation of miR-25 expression in palmitate-treated INS-1 cells (Fig. 1B). We further confirmed that palmitate also increased miR-25 expression in human primary islets (Fig. 1C). Treatment of INS-1 cells with tunicamycin triggered a more than two-fold increase in miR-25 expression in human islets (Fig. 1 D). These results strongly suggested that diabetes pathogenic factors, like IL-1 β , upregulated miR-25 expression through ER stress pathways.

Overexpression of miR-25 suppresses insulin biosynthesis

We also examined the influence of miR-25 overexpression on β -cell function. GSIS and KSIS tests showed a decreased insulin content at 48 h after transfection (Fig. 2C and D), with a more marked effect apparent in the INS-1 cells than in the MIN6 cells (Fig. 2C and D). However, neither basal insulin secretion nor GSIS was affected by miR-25 overexpression (Fig. 2A and B), nor was KSIS affected. These results indicate that miR-25 exerts its effects on β -cells by inhibiting insulin biosynthesis, rather than affecting β -cell insulin secretion. This contradicts the known inhibitory effects of IL-1 β on both insulin biosynthesis and secretion, suggesting that miR-25 functions in a different way than IL-1 β .

Previous research has demonstrated that β -cell maturation genes have a significant effect on insulin biosynthesis [15, 16]. Transcription factors of insulin, including PDX1, MAFA, and NEUROD1, play a critical role in maturation. We further examined the volatility of gene expression levels under miR-25 regulation as a way to identify a possible regulation path by analyzing related genes from the aspect of RNA transcription.

Transfection with miR-25 led to a marked decrease in mRNA levels of *Ins2*, *Pdx1*, *Glut2*, and *Gck*, and a slightly smaller drop in *Ins1* and *Mafa* mRNA levels (Fig. 2E). *Ins2* is the only insulin gene in human beings; therefore, repression of *Ins2* transcription by miR-25 overexpression may be an important driver of the diabetes process. By contrast, the *Neurod1* mRNA levels remained unchanged. Further western blot analysis revealed that overexpression of miR-25 reduced the levels of PDX1, MAFA, and NEUROD1 proteins (Fig. 2F and G). This finding suggested that the effect of miR-25 on NEUROD1 is mainly post-transcriptional. Taken together, upregulation of miR-25 may suppress insulin biosynthesis by inhibiting β -cell maturation genes. This mechanism differs in part from the mechanism by which IL-1 β impairs β -cell function.

Overexpression of miR-25 increases ER-stress–induced β -cell apoptosis

β -cell dysfunction caused by diabetes pathogenic factors is usually accompanied by increased apoptosis. We therefore tested the influence of miR-25 overexpression on β -cell apoptosis by transfecting MIN6 and INS-1 cells with miR-25 mimics. Cell viability decreased (Fig. 3A and D), suggesting that miR-25 overexpression reduced β -cell proliferation. Subsequent treatment of INS-1 cells with IL-1 β or palmitate also decreased cell viability (Fig. 3B and C). The observed induction of miR-25 by IL-1 β or palmitate led us to consider that miR-25 is at least one of the important mediators of β -cell apoptosis caused by diabetic pathogenic factors. Interestingly, INS-1 cells transfected with miR-25 and IL-1 β or palmitate showed a further drop in cell viability (Fig. 3B and C). These findings led us to assume that miR-25 induced β -cell apoptosis through a compensatory pathway.

We also confirmed the increase in cell apoptosis at the morphological and molecular levels. Hoechst staining for apoptotic cells in both cell lines showed significant increases in cells with overexpression of miR-25 (Fig. 3E and F). Western blot analysis also revealed that upregulation of miR-25 increased the cleaved PARP1 and cleaved CASP-3 levels and decreased the complete PARP levels (Fig. 3E and F).

Cleaved CASP-3 is the active form of CASP-3 and cleaves PARP1, resulting in cell apoptosis. In other words, miR-25 promotes cell apoptosis through a caspase 3/PARP-1 – dependent pathway.

Validation of the miR-25 target genes

We then investigated the potential mechanisms by which an upregulation of miR-25 expression might trigger β -cell death. Target genes were predicted, and gene ontology analysis identified several potential apoptosis-related target genes. We recognized that miR-25-3p and miR-92b-3p have the same seed sequence, so we simultaneously assessed the conservation of miR-25-3p and miR-92b-3p binding sites. Ultimately, MCL1 and NEUROD1 were selected as suitable candidates.

We demonstrated our prediction of the target gene binding sites by cotransfecting INS-1 cells, as described in the drawing note. As anticipated, the relative luciferase activity declined in response to miR-25 overexpression (Fig. 4C and D), and this effect was reversed by mutations of the miR-25 binding sites in the 3'UTR sequence of MCL1 or NEUROD1. Western blotting analysis also revealed a decrease in MCL1 and NEUROD1 in response to miR-25 overexpression in INS-1 cells (Fig. 4E). Measurements of the protein levels of PDX1 and MAFA also confirmed a similar decrease in response to miR-92b overexpression (Fig. 4F).

β -cell apoptosis induced by miR-25 is reversed by overexpression of MCL1

The regulatory effect of miR-25 on MCL1 has been described previously, but a direct relationship between the reduction in MCL1 and the effect of miR-25 on β -cell apoptosis has not been demonstrated. Both Hoechst staining and TUNEL staining showed a significant increase in apoptosis in β -cells transfected with miR-25 mimics. However, this situation was reversed by overexpression of MCL1 (Fig. 5B and C) and was reflected by the partial reversal of the increase in pyknotic cells and TUNEL-positive β -cells that had resulted from transfection of miR-25 (Fig. 5D and E). These findings indicated that at least part of the detrimental impact of miR-25 expression is mediated by a reduced expression of MCL1, a crucial antiapoptotic protein.

Western blotting analysis also revealed a decrease in cleaved PARP and cleaved CASP-3 and an increase in MAFA following overexpression of MCL1 in β -cells transfected with miR-25 mimics (Fig. 5A). The observed reduction in apoptosis markers agreed with the Hoechst and TUNEL staining results and further established MCL1 as an important mediator in miR-25 regulated β -cell apoptosis in an inflammatory environment. However, one notable observation was that MCL1 overexpression did not restore the levels of NEUROD1 protein.

Discussion

In this study, we identified that diabetic pathogenic factors, such as IL-1 β and palmitate, upregulated miR-25 expression in human islets and rat insulinoma INS-1 cells. The increased amounts of miR-25 bind to the 3'-UTR of Neurod1 and Mcl1 and suppress the amounts of NEUROD1 and MCL1 proteins. The miR-

25/NEUROD1 axis then inhibits insulin biosynthesis by transcriptional regulation of β -cell specific genes, while the miR-25/MCL1 axis triggers β -cell apoptosis in a caspase 3/PARP1-dependent manner. The regulatory role of miR-25 is conserved among species, and we confirmed this in both INS-1 and MIN6 cells. Thus, miR-25 instigates a stepwise impairment of β -cell function during the pathogenesis of diabetes mellitus.

The increased miR-25 expression in β cells treated with proinflammatory IL-1 β was discovered using an Affymetrix miRNA ChIP. Previous research has demonstrated that both IL-1 β and palmitate induce PERK phosphorylation, which then phosphorylates eIF2 α to induce cell apoptosis by an ER stress-dependent mechanism [17-19]. We further confirmed that palmitate and tunicamycin, two classic ER stress activators, further enhanced the miR-25 level in human islets, indicating a broad regulatory effect of miR-25 under ER stress conditions.

The molecular mechanisms upregulating miR-25 expression require further investigation. Currently, some theories have been put forward for the mechanism of miR-25 regulation. For example, inhibition of the expression of DNA methyltransferases (DNMTs), and TET1 protein dependent pathway [20]. Both ectopic overexpression of miR-25 and treatment with IL-1 β result in defective insulin biosynthesis and increased β -cell apoptosis. However, IL-1 β also inhibits GSIS due to enhanced NO production and dampened mitochondrial ATP production [21, 22]. GSIS represents an early damage event in β cells, but GSIS defects were barely affected by increased miR-25 expression. We also tested KSIS, which causes insulin release by membrane depolarization. The lack of any alteration in GSIS and KSIS abilities by miR-25 obviously excludes the involvement of insulin secretion due to soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and ATP production from mitochondrial bioenergy processes.

We also observed a further inhibitory effect on cell viability by miR-25 elevation by IL-1 β treatment of INS-1 cells. This hints that miR-25 and IL-1 β may trigger β -cell apoptosis through different pathways. The differences largely involve the activation of NF- κ B/iNOS/COX2 pathway by IL-1 β but not by miR-25 (data not shown). As a downstream molecule of IL-1 β , miR-25 may function in a compensatory pathway. Both miR-34a and miR-153 inhibit insulin secretion by targeting several SNAREs to inhibit insulin granule docking and fusion, and they promote β -cell apoptosis by targeting Bcl2 [23, 24]. Alternatively, miR-146a, which is upregulated by IL-1 β /NF- κ B, causes either impairment or negative feedback protective effects through different targets, including Irak1 and Traf6 [25]. Here, we show that miR-25, unlike other IL-1 β -regulated miRNAs, specifically targets NEUROD1 and MCL1 to function as a unique supplement pathway to β -cell dysfunction during diabetes progression.

NEUROD1 binds to the E-box element in the promoter region of genes and regulates comprehensive gene expressions that play important roles in both pancreatic endocrine and nervous system development. Deletion of Neurod1 results in defective pancreatic islet morphogenesis and an early development of diabetes [26]. Indeed, NEUROD1, in combination with PDX1 and MAFA, determines the development and functional maturation of β cells by regulating insulin related genes, such as Glut2 and Gck[26]. We previously reported that diabetes pathogenic factors upregulate miR-24 to inhibit insulin secretion and β -

cell growth via targeting Neurod1 and Hnf1a [12]. However, elevation of miR-24 does not induce β -cell apoptosis but instead guides β -cell dedifferentiation to avoid ER-stress insults [28]. The regulatory role between miR-24/ NEUROD1 might explain the pro-apoptotic effect of NEUROD1 under ER-stress conditions.

MCL1, as a BCL-2 homologue, is known to function as an anti-apoptotic protein by sequestering the multi-BH domain pro-apoptotic BAX/BAK proteins and the pro-apoptotic BH3-only proteins, such as BIM and PUMA [29]. Some reports have shown that MCL1 is decreased by proinflammatory cytokines and ER stressors and that its decrease leads to β -cell apoptosis in both rodent and human islets. Importantly, relatively low levels of cytokines were able to reduce MCL1 protein levels and promote apoptosis in the human EndoC β H1 β -cell line, suggesting that MCL1 reduction is an early event in the development of diabetes. Indeed, β -cell-specific MCL1 knockout (β MCL1 KO) mice are prone to multiple low-dose streptozotocin-induced islet inflammation [30].

Palmitate and ER stressors inhibit MCL1 translation through eIF2 α phosphorylation, whereas cytokines reduce MCL1 protein amounts through combination of translation inhibition and JNK pathway which targets MCL1 for degradation [18]. Cytokines also upregulate miR-29 expression to repress MCL1 translation, leading to β -cell demise in a type 1 diabetic mouse model [18]. Here, we also found that miR-25 could decrease MCL1 protein levels. Furthermore, recovery of MCL1 expression rescued miR-25-induced β -cell apoptosis, indicating that MCL1 also contributes to the pathogenesis of type 2 diabetes. Decreased MCL1 leads to proapoptotic Bax translocation from the cytoplasm to the mitochondria and gives rise to mitochondrial outer membrane permeabilization, followed by cytochrome c release, caspase-3 cleavage, PARP1 cleavage, and, ultimately, cell apoptosis, consistent with our current data [18, 32].

Our study also complements previous research that demonstrated that a lack of miRNA-25 in β -cells could hasten cell apoptosis and loss of cell function, although the effect was mild. This suggests that both excessive and absent expression of miRNAs can cause damage to cells [34]. Compensatory elevation of other members in the same miRNA “family” may be the explanation. We also confirmed that miR-92b could regulate the expression of miR-25 target genes due to their common seed sequences. The miR-106b-25 family is actually quite important in normal endocrine function, as losses may cause endocrine failure [35]. For example, a severe defect in β -cell division was observed in miR-17-92/miR-106b-25 double knockout mice [35]. Studies by Munish Kumar et al. have also revealed that miR-25 expression repressed the transcription and expression of the p53 gene, a famous tumor suppressor gene, along with transactivation genes, such as p21, Bax, Puma, and Gadd45a, thereby hindering cell death. Overall, the available research suggests that β cells might have more complex regulatory networks that partly rely on different environmental stimulation signals, but this possibility requires further correlational research.

The establishment of commercial human pancreas β -cell lines has not yet been completely successful, despite much effort invested over the past few decades [36, 37]. Therefore, access to functional human islets or derived β -cell lines is limited. Rodent insulinoma cell lines are often used for research on insulin

production and cell survival related to miRNA studies. We restricted our study to the INS-1 and MIN6 β -cell lines due to their relatively wide acceptance. One clinical research report involving two T1DM cohorts has shown that T1DM children have significantly increased levels of miR-25 in their serum, and that this level negatively correlates with residual β -cell function [38]. Given the diversity in different races and species, more animal and human experiments remain to be done in the future.

In summary, our study reveals that miR-25 can be induced by pathogenic factors of diabetes, and that it drives β -cell failure by directly repressing NEUROD1 and MCL1 production. The findings further elaborate the regulatory networks involving miRNAs in control of islet β -cell survival and function. The results also broaden our understanding of the pathophysiological development of diabetes and point to new strategic directions for prevention of the loss of β -cell mass during the development of diabetes mellitus.

Declarations

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Conflicts of interests

The authors declare that there is no duality of interest associated with this manuscript.

References

- [1]. Eizirik, D.L., L. Pasquali and M. Cnop, Pancreatic beta-cells in type 1 and type 2 diabetes mellitus: different pathways to failure. *Nat Rev Endocrinol*, 2020. 16(7): p. 349-362.
- [2]. Rodriguez-Comas, J., et al., Stress-Induced MicroRNA-708 Impairs beta-Cell Function and Growth. *Diabetes*, 2017. 66(12): p. 3029-3040.
- [3]. Huang, Q., et al., Glucolipotoxicity-Inhibited miR-299-5p Regulates Pancreatic beta-Cell Function and Survival. *Diabetes*, 2018. 67(11): p. 2280-2292.
- [4]. Oh, Y.S., Mechanistic insights into pancreatic beta-cell mass regulation by glucose and free fatty acids. *Anat Cell Biol*, 2015. 48(1): p. 16-24.
- [5]. Remedi, M.S. and C. Emfinger, Pancreatic β -cell identity in diabetes. *Diabetes, Obesity and Metabolism*, 2016. 18: p. 110-116.
- [6]. Talchai, C., et al., Pancreatic beta cell dedifferentiation as a mechanism of diabetic beta cell failure. *Cell*, 2012. 150(6): p. 1223-34.

- [7]. Bartel, D.P., *MicroRNAs: Genomics, Biogenesis, Mechanism, and Function*. 2004, Elsevier Inc: United States. p. 281-297.
- [8]. Guo, H., et al., Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature*, 2010. 466(7308): p. 835-840.
- [9]. Kalis, M., et al., Beta-cell specific deletion of Dicer1 leads to defective insulin secretion and diabetes mellitus. *PLoS One*, 2011. 6(12): p. e29166.
- [10]. Yu, Y., et al., miRNA-mRNA profile and regulatory network in stearic acid-treated beta-cell dysfunction. *J Endocrinol*, 2020. 246(1): p. 13-27.
- [11]. Guo, J., et al., Palmitate-induced inhibition of insulin gene expression in rat islet beta-cells involves the ceramide transport protein. *Cell Physiol Biochem*, 2010. 26(4-5): p. 717-28.
- [12]. Zhu, Y., et al., MicroRNA-24/MODY gene regulatory pathway mediates pancreatic beta-cell dysfunction. *Diabetes*, 2013. 62(9): p. 3194-206.
- [13]. Zhu, Y., et al., Inhibition of the receptor for advanced glycation endproducts (RAGE) protects pancreatic beta-cells. *Biochem Biophys Res Commun*, 2011. 404(1): p. 159-65.
- [14]. Li, Y., et al., Inhibition of tumor suppressor p53 preserves glycation-serum induced pancreatic beta-cell demise. *Endocrine*, 2016. 54(2): p. 383-395.
- [15]. Salinno, C., et al., beta-Cell Maturation and Identity in Health and Disease. *Int J Mol Sci*, 2019. 20(21).
- [16]. Spaeth, J.M., et al., Defining a Novel Role for the Pdx1 Transcription Factor in Islet beta-Cell Maturation and Proliferation During Weaning. *Diabetes*, 2017. 66(11): p. 2830-2839.
- [17]. Cnop, M., et al., Endoplasmic reticulum stress and eIF2alpha phosphorylation: The Achilles heel of pancreatic beta cells. *Mol Metab*, 2017. 6(9): p. 1024-1039.
- [18]. Allagnat, F., et al., Mcl-1 downregulation by pro-inflammatory cytokines and palmitate is an early event contributing to beta-cell apoptosis. *Cell Death Differ*, 2011. 18(2): p. 328-37.
- [19]. Rozpedek, W., et al., The Role of the PERK/eIF2alpha/ATF4/CHOP Signaling Pathway in Tumor Progression During Endoplasmic Reticulum Stress. *Curr Mol Med*, 2016. 16(6): p. 533-44.
- [20]. Wu, M.Z., et al., miR-25/93 mediates hypoxia-induced immunosuppression by repressing cGAS. *Nat Cell Biol*, 2017. 19(10): p. 1286-1296.
- [21]. Elouil, H., et al., High glucose and hydrogen peroxide increase c-Myc and haeme-oxygenase 1 mRNA levels in rat pancreatic islets without activating NFkappaB. *Diabetologia*, 2005. 48(3): p. 496-505.

- [22]. Thomaidou, S., A. Zaldumbide and B.O. Roep, Islet stress, degradation and autoimmunity. *Diabetes Obes Metab*, 2018. 20 Suppl 2: p. 88-94.
- [23]. Lin, X., et al., Downregulation of Bcl-2 expression by miR-34a mediates palmitate-induced Min6 cells apoptosis. *J Diabetes Res*, 2014. 2014: p. 258695.
- [24]. Sun, Y., et al., Inhibition of miR-153, an IL-1beta-responsive miRNA, prevents beta cell failure and inflammation-associated diabetes. *Metabolism*, 2020. 111: p. 154335.
- [25]. Taganov, K.D., et al., NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci U S A*, 2006. 103(33): p. 12481-6.
- [26]. Naya, F.J., et al., Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice. *Genes Dev*, 1997. 11(18): p. 2323-34.
- [27]. Fu, Z., E.R. Gilbert and D. Liu, Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. *Curr Diabetes Rev*, 2013. 9(1): p. 25-53.
- [28]. Zhu, Y., et al., MicroRNA-24 promotes pancreatic beta cells toward dedifferentiation to avoid endoplasmic reticulum stress-induced apoptosis. *J Mol Cell Biol*, 2019. 11(9): p. 747-760.
- [29]. Senichkin, V.V., et al., Molecular Comprehension of Mcl-1: From Gene Structure to Cancer Therapy. *Trends Cell Biol*, 2019. 29(7): p. 549-562.
- [30]. Meyerovich, K., et al., MCL-1 Is a Key Antiapoptotic Protein in Human and Rodent Pancreatic beta-Cells. *Diabetes*, 2017. 66(9): p. 2446-2458.
- [31]. Roggli, E., et al., Changes in microRNA expression contribute to pancreatic beta-cell dysfunction in prediabetic NOD mice. *Diabetes*, 2012. 61(7): p. 1742-51.
- [32]. Delbridge, A.R., et al., Thirty years of BCL-2: translating cell death discoveries into novel cancer therapies. *Nat Rev Cancer*, 2016. 16(2): p. 99-109.
- [33]. Jacovetti, C., et al., Postnatal beta-cell maturation is associated with islet-specific microRNA changes induced by nutrient shifts at weaning. *Nat Commun*, 2015. 6: p. 8084.
- [34]. Eliasson, L. and R. Regazzi, Micro(RNA) Management and Mismanagement of the Islet. *J Mol Biol*, 2020. 432(5): p. 1419-1428.
- [35]. Mandelbaum, A.D., et al., miR-17-92 and miR-106b-25 clusters regulate beta cell mitotic checkpoint and insulin secretion in mice. *Diabetologia*, 2019. 62(9): p. 1653-1666.
- [36]. Zhao, Y.F., et al., Cholesterol induces mitochondrial dysfunction and apoptosis in mouse pancreatic beta-cell line MIN6 cells. *Endocrine*, 2010. 37(1): p. 76-82.

[37]. Tuo, Y., et al., Long-term exposure of INS-1 rat insulinoma cells to linoleic acid and glucose in vitro affects cell viability and function through mitochondrial-mediated pathways. *Endocrine*, 2011. 39(2): p. 128-38.

[38]. Nielsen, L.B., et al., Circulating levels of microRNA from children with newly diagnosed type 1 diabetes and healthy controls: evidence that miR-25 associates to residual beta-cell function and glycaemic control during disease progression. *Exp Diabetes Res*, 2012. 2012: p. 896362.

Figures

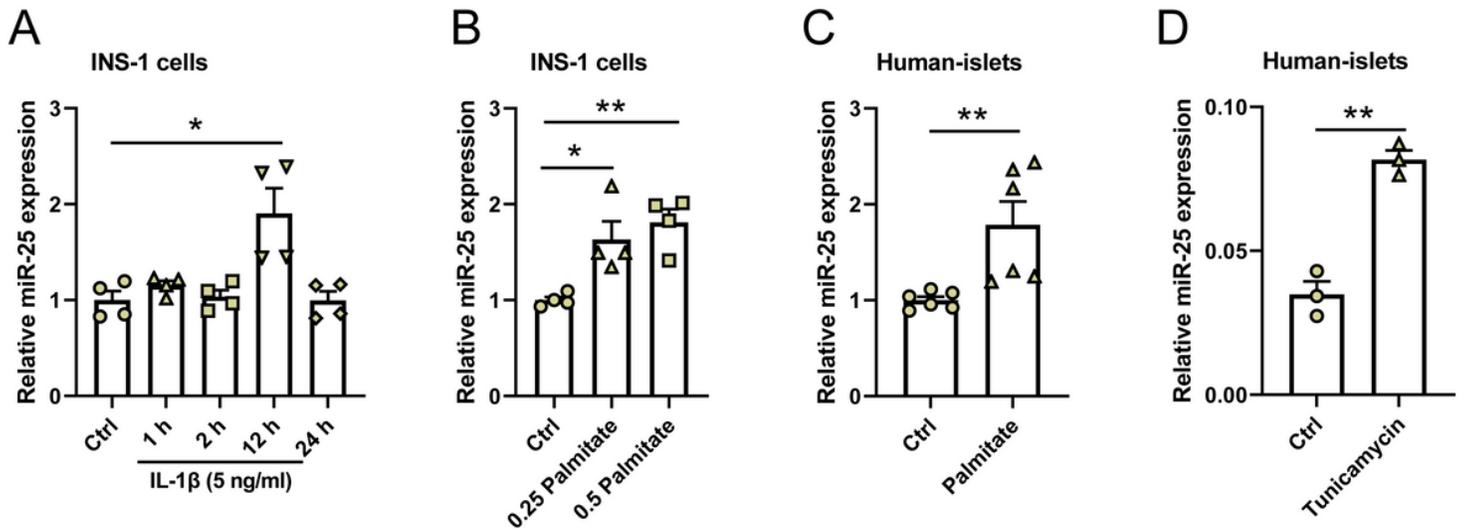


Figure 1

Levels of miR-25 is increased by diabetes pathogenic factors in human islets and INS-1 cells. A: INS-1 cells were exposed to Ctrl or IL-1β (5 ng/ml), when expression of miR-25 was measured by qRT-PCR after exposure for 1h, 2h, 12h and 24h. *p < 0.05 vs. Ctrl (n=4). B: INS-1 cells were exposed to Ctrl or 0.25 mmol/L or 0.5 mmol/L palmitate for 24h, when expression of miR-25 was measured by qRT-PCR. *p < 0.05 or **p < 0.01 vs. Ctrl (n=4). C and D: Human-islets were isolated and incubated with 0.5 mmol/l palmitate or 10 μg/ml tunicamycin for 24h, when expression of miR-25 was measured by qRT-PCR. **p < 0.01 vs. Ctrl (n=6 in C and n=3 in D).

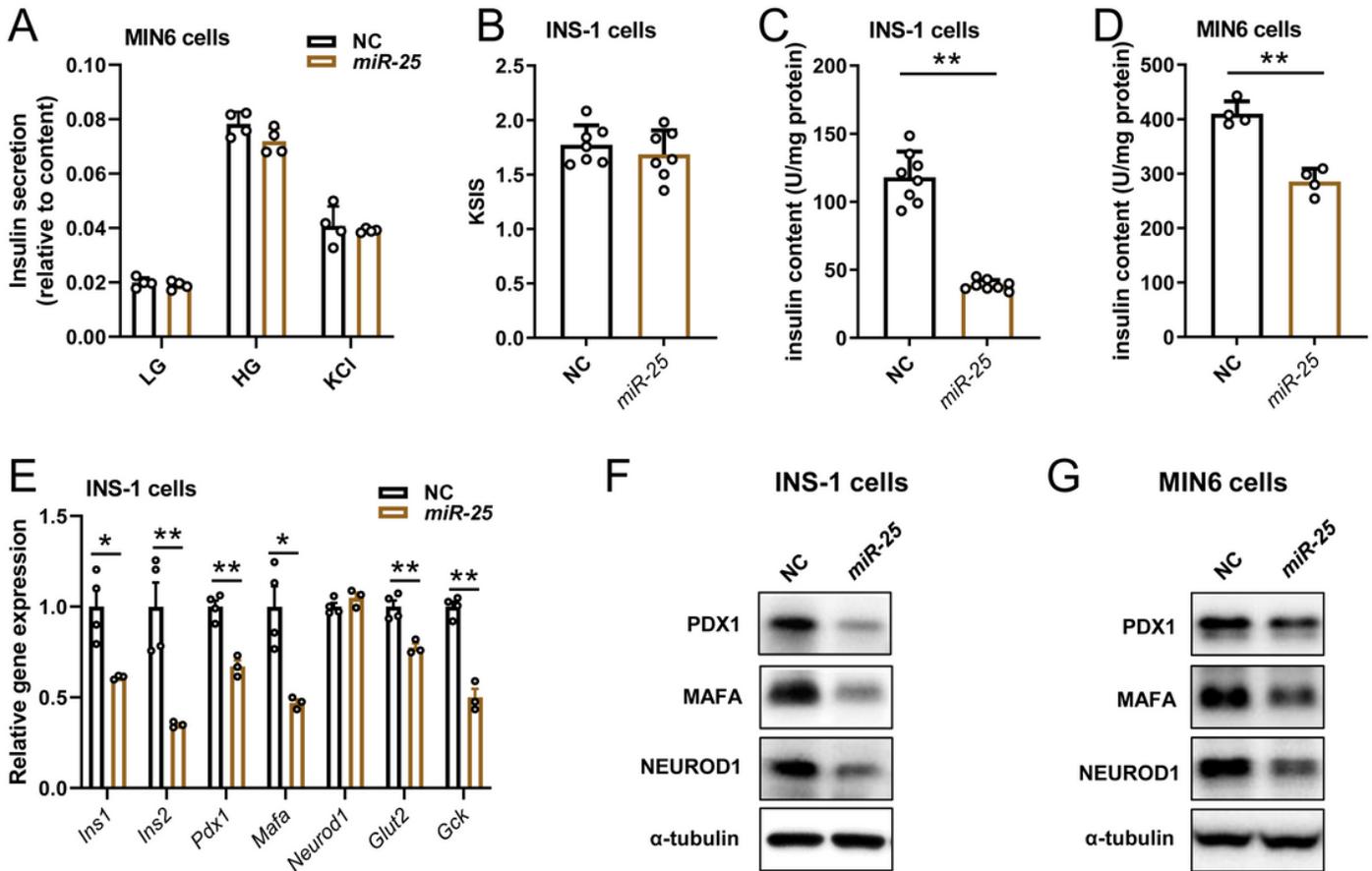


Figure 2

Overexpression of miR-25 suppresses insulin biosynthesis via inhibiting β -cell maturation genes. A: MIN6 cells were transfected with a control RNA duplex (NC) or with duplexes corresponding to the mature forms of miR-25. Two days later, the cells were exposed to low glucose (LG), high glucose (HG) or KCl for 1h, and then insulin secretion was assessed by GSIS assay (n=4). B: INS-1 cells were transfected with a control RNA duplex or with duplexes corresponding to the mature forms of miR-25 for 48h, and then insulin secretion was assessed by K SIS assay (n=7). C and D: INS-1 cells or MIN6 cells were transfected with a control RNA duplex or with duplexes corresponding to the mature forms of miR-25 for 48h. After GSIS or K SIS assay, insulin content was extracted by acid ethanol (n=8 in C and n=4 in D). E: INS-1 cells were transfected with a control RNA duplex or with duplexes corresponding to the mature forms of miR-25 for 48h, and then mRNA levels of insulin I (Ins1), insulin II (Ins2), pancreatic and duodenal homeobox 1 (Pdx1), MAF bZIP transcription factor A (Mafa), neuronal differentiation 1 (Neurod1), glucose transporter 2 (Glut2) and glucokinase (Gck) were measured by qRT-PCR (n=3-4). F and G: Meanwhile, protein levels of PDX1, MAFA, NEUROD1 and α -tubulin were measured by western blotting in INS-1 cells and MIN6 cells. *p < 0.05 vs.NC (E) or **p < 0.01 vs.NC (C, D and E).

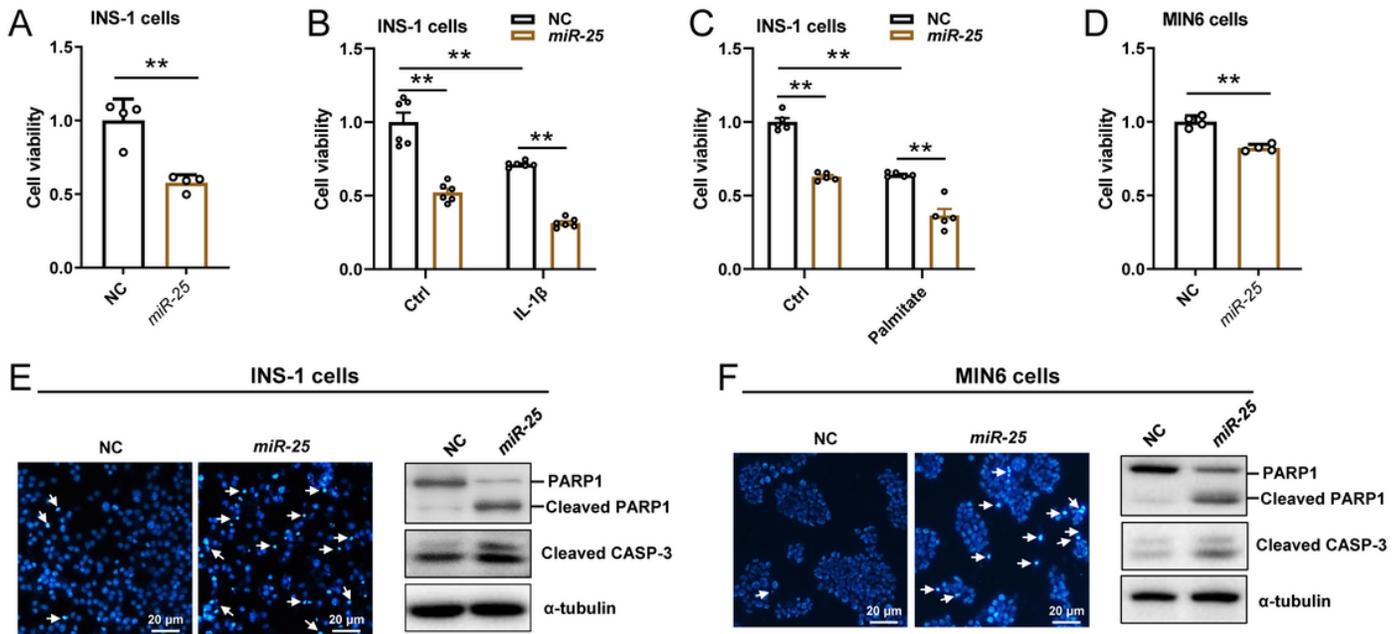


Figure 3

Overexpression of miR-25 synergistically increases ER-stress induced β -cell apoptosis. A and D: INS-1 cells or MIN6 cells were transfected with a control RNA duplex (NC) or with duplexes corresponding to the mature forms of miR-25 for 48 h, and then cell viability was assessed by CCK-8 assay (n=4). B and C: INS-1 cells were transfected with a control RNA duplex (NC) or with duplexes corresponding to the mature forms of miR-25 for 48 h followed by IL-1 β or palmitate treatment for 24 h, and then cell viability was assessed by CCK-8 assay (n=6 in B and n=5 in C). E and F: INS-1 cells or MIN6 cells were transfected with a control RNA duplex (NC) or with duplexes corresponding to the mature forms of miR-25. After two days, Hoechst staining for apoptotic cells was carried out, and protein levels of poly(ADP-ribose) polymerase1 (PARP1), cleaved PARP1, cleaved caspase-3 (cleaved CASP-3) and α -tubulin were measured by Western Blotting. **p < 0.01 vs. NC (A, B, C and D) or vs. Ctrl (B and C).

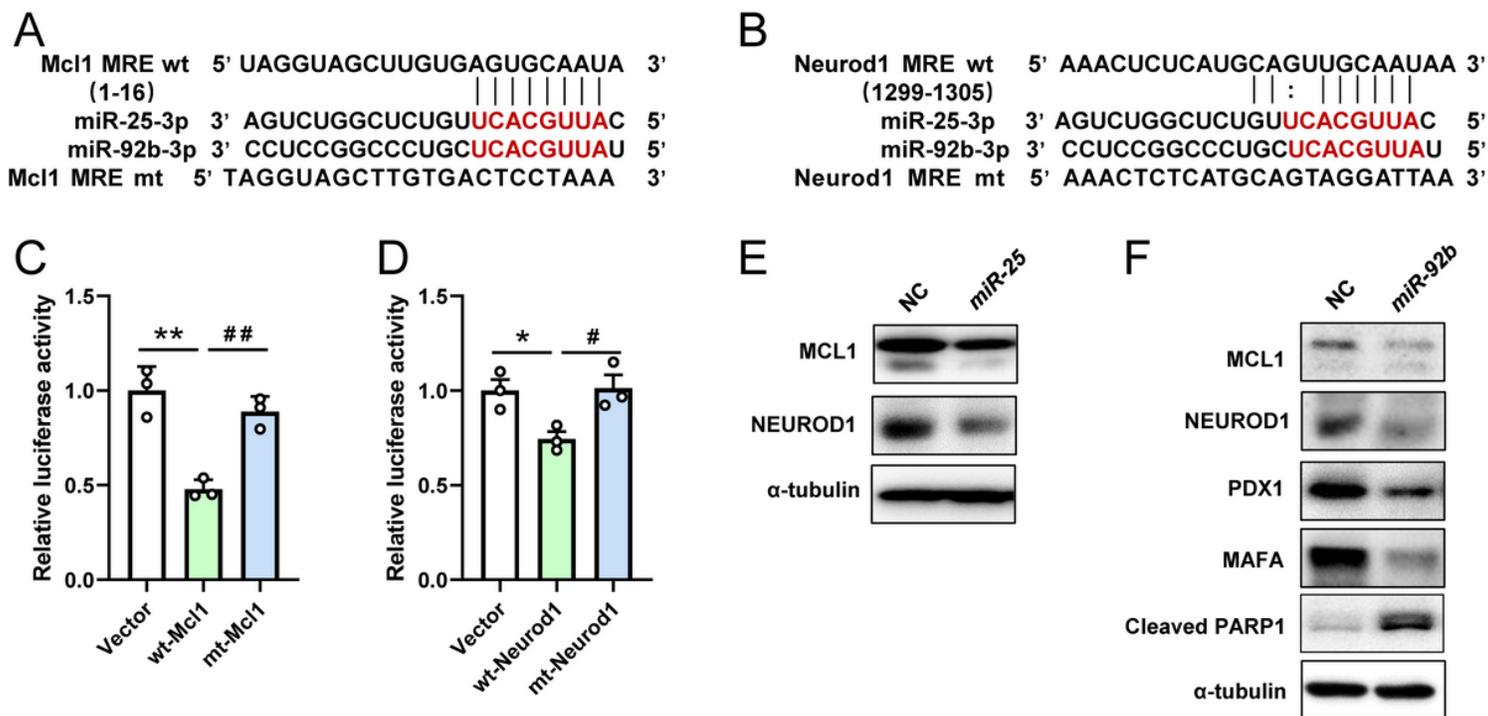


Figure 4

miR-25/miR-92 binds to 3'UTR of Mcl1 and Neurod1 message RNAs. A and B: The 3'UTR regions of Myeloid cell leukemia-1 (Mcl1) and Neurod1 genes predicted to include miR-299-5p MREs are aligned with miR-25-3p and miR-92b-3p, and both wild-type (wt) and mutant (mt) sequences are listed. C and D: Luciferase reporter assay was carried out 24 h after INS-1 cell cotransfection with wild type (wt) or mutant type (mt) reporter plasmids containing the 3'UTR sequence of Mcl1 or Neurod1, PRL-SV40 plasmid and NC or miR-25 (n=3). E and F: INS-1 cells were transfected with a control RNA duplex (NC) or with duplexes corresponding to the mature forms of miR-25 or miR-92b for 48h, and then protein levels of MCL1, NEUROD1, PDX1, MAFA, Cleaved PARP1 and α -tubulin were by western blotting. *p < 0.05, **p < 0.01 vs. Vector (C and D); #p < 0.05, ##p < 0.01 vs. wt-Neurod1 or vs. wt-MCL1 (C and D).

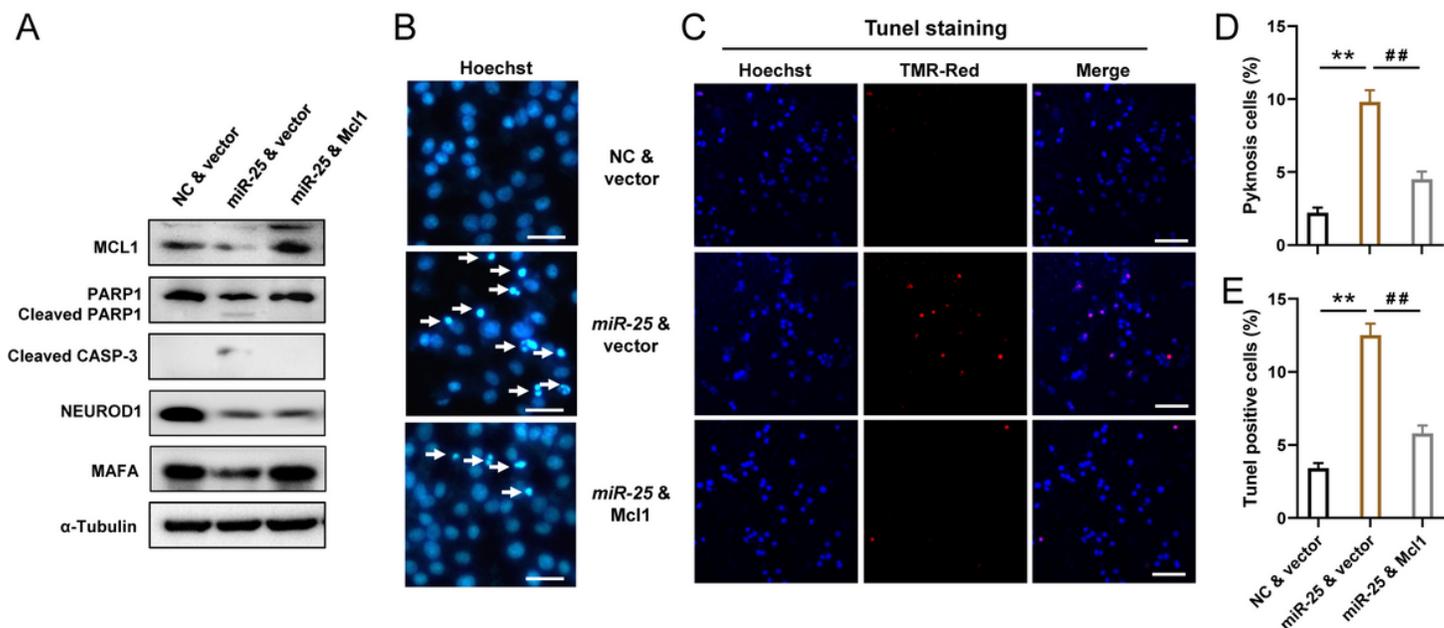


Figure 5

Overexpression of MCL1 protects from miR-25 induced β -cell apoptosis. A: INS-1 cells were cotransfected with NC and vector or miR-25 and vector or miR-25 and Mcl1 for 48h, and then protein levels of MCL1, PARP1, Cleaved PARP1, cleaved CASP-3, NEUROD1, MAFA and α -Tubulin were measured by western blotting. B: INS-1 cells were cotransfected with NC and vector or miR-25 and vector or miR-25 and MCL1 for 48h, and then Hoechst 33342 was performed. C: INS-1 cells were cotransfected with NC and vector or miR-25 and vector or miR-25 and Mcl1 for 48h, and then TUNEL staining was performed. D: INS-1 cells with pyknotic nuclei in figure C were counted. E: TUNEL-positive cells in figure C were counted. ** $p < 0.01$ vs. NC & Vector (D and E); ## $p < 0.01$ vs. miR-25 & Vector (D and E).