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Original Research

Bone Marrow Mesenchymal Stem Cells-Derived miR-21-5p Protects Grafted Islets Against Apoptosis by Targeting PDCD4

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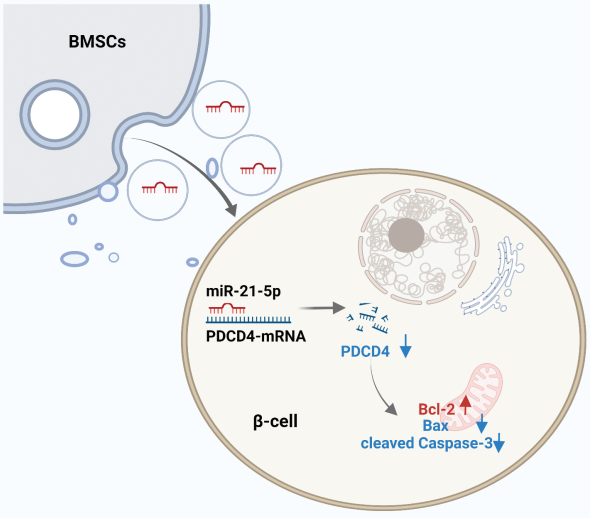
Abstract

The apoptosis of grafted islets is an urgent problem due to the high rate of islet loss soon after transplantation. MicroRNA-21-5p (miR-21-5p) is an essential mediator of bone marrow mesenchymal stem cells-derived exosomes (BMSCs-Exo) during anti-apoptosis, but its effect and the underlying molecular mechanism in islet transplantation remain partially understood. Here, we found that miR-21-5p could be delivered to islet cells via BMSCs-Exo. Subsequently, we demonstrated that miR-21-5p overexpression reduced apoptosis in islets and INS-1 cells, whereas miR-21-5p inhibition enhanced apoptosis. A mechanistic analysis involving RNA sequencing and bioinformatic analysis was performed to determine the interaction between miR-21-5p and its target gene programmed cell death 4 (PDCD4), which was further verified by a dual luciferase assay. In vivo, the grafted islets overexpressing miR-21-5p showed a higher survival rate, better insulin secretion function, and a lower apoptosis rate. In conclusion, these results demonstrated that miR-21-5p from BMSCs-Exo protects against the apoptosis of grafted islets by inhibiting PDCD4 expression. Hence, miR-21-5p can be used as a cell-free therapeutic agent to minimize β-cell apoptosis at the early stage of islet transplantation.

Key words: islet transplantation; bone marrow mesenchymal stem cells; exosomes; miR-21-5p; PDCD4; apoptosis.

Graphical Abstract



BMSCs-derived exosomal microRNA-21-5p reduces the early apoptosis of grafted islets by targeting PDCD4.

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Significance Statement

This study demonstrated that bone marrow mesenchymal stem cells could deliver exosomes containing miR-21-5p into islet cells. The study also found that miR-21-5p overexpression attenuated apoptosis of islets and rat insulinoma (INS-1) cells, while miR-21-5p inhibition significantly promoted apoptosis. The upregulated miR-21-5p directly binds to the 3′UTR of PDCD4, thereby reducing apoptosis in transplanted islets. This study confirms the results of previous studies, suggesting that miR-21-5p can be used as a therapeutic agent to minimize β-cell apoptosis in the early stage of islet transplantation and thus improve transplantation outcome.

Introduction

Type 1 diabetes mellitus (T1DM) results in hyperglycemia due to absolute insulin insufficiency and is characterized by pancreatic islet inflammation and progressive β-cell loss via apoptosis.[1,2] Islet transplantation is considered one of the most effective treatments for insulin-dependent diabetes mellitus.[3] Over the past 20 years, human islet transplantation has been a great success.[4] Unfortunately, islet viability is one of the most critical challenges associated with islet transplantation.[5] Isolated islets suffer from chronic hypoxia and ischemia at an early stage, which lead to increased apoptotic β-cell death.[6] Therefore, strategies that promote islet survival may be effective in successful islet transplantation.

Bone marrow mesenchymal stem cells (BMSCs), which are widely cotransplanted with islets, can promote islet survival and function in vitro and in vivo, but the mechanism has not been fully clarified.[7] This effect was also shown in our previous study.[8] BMSCs can release a large number of exosomes, which can carry many miRNAs, and thereby affect many pathophysiological functions of recipient cells.[9] MicroRNAs (miRNAs), a class of noncoding microRNAs with a length of approximately 22 bases, can modulate gene expression by inhibiting mRNA translation or inducing mRNA degradation.[10]

Among these microRNAs, miR-21-5p has been one of the most extensively studied. Increasing evidence shows that miR-21-5p is associated with apoptosis.[11,12] However, the biological roles of miRNAs may be cell specific. Inhibiting miR-21-5p can induce apoptosis in glioma cells[12]; and miR-21-5p can increase HeLa cell apoptosis.[13]

The effects of miRNAs on islets and DM are being increasingly appreciated.[14] For example, BMSCs-derived exosomal miR-146a improves pancreatic islet function and reverses β-cell dedifferentiation.[15] However, the biological role of miR-21-5p in islet transplantation has not been examined. The present study aimed to investigate the potential role of miR-21-5p in apoptosis in transplanted islets and elucidate the underlying mechanism. Our studies demonstrated that miR-21-5p could significantly reduce the early apoptosis of grafted islets, which indicates that targeting miR-21-5p is a potential therapeutic strategy to improve the efficacy of islet transplantation.

Materials and Methods

Pancreatic Islet Isolation and Cell Culture

Male Lewis rats were obtained from the Experimental Animal Center of Xi'an Jiaotong University, Xi'an. This study was approved by the Xi'an Jiaotong University Committee on Animal Care and was conducted according to the guidelines (NO.2019-085). Rat pancreatic islets were isolated from male Lewis donors (250-300 g). Pancreatic islets were isolated and purified using the collagenase P (1 mg/mL, Roche) digestion method combined with the histopaque-1083 gradient (Sigma-Aldrich) purification method, as previously described.[16] The islet yield and purity were determined by dithizone (DTZ, Sigma-Aldrich) staining. The rat islets were cultured overnight under standard culture conditions in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin before use.

A rat insulinoma cell line (INS-1 cells) was provided by the Cell Centre of Shanghai Institutes for Biological Sciences (Shanghai, China), and the cells were cultured in RPMI 1640 medium (Sigma-Aldrich). The media was supplemented with 10% FBS, 1% penicillin/streptomycin, and 50 μM β-mercaptoethanol. The cultures were incubated at 37 °C in a humidified chamber with 5% CO_2.

Isolation, Culture, and Characterization of BMSCs

sThe isolation, culture, and passage of rat BMSCs from 7-day-old Lewis rats were performed as previously described. Briefly, under sterile conditions, a syringe was used to rinse the bone marrow. BMSCs were cultured as monolayers in mesenchymal stem cell growth medium (RASMX-01001, Cyagen). The cells were digested and passaged at a ratio of 1:3 when they reached >90% confluence. BMSCs from passage 3 to passage 5 were used in the experiments.

To identify BMSCs, the immunophenotype was examined by flow cytometry. The surface molecular markers of BMSCs were identified individually by staining with fluorescein isothiocyanate (FITC)-labeled anti-CD29, phycoerythrin (PE)-labeled anti-CD90, FITC-labeled anti-CD45 and PE-labeled anti-CD11b/c monoclonal antibodies (eBioscience) using a FACS Calibur instrument (BD Biosciences). The differentiation of BMSCs to adipocytes and osteoblasts was then examined by Oil Red O (G1262, Solarbio) or Alizarin Red (G1450, Solarbio) staining, respectively.

Isolation and Characterization of BMSC-Derived Exosomes

Upon reaching 70%-80% confluence, BMSCs at P3 were washed twice with phosphate-buffered saline (PBS) and then cultured in an exosome-depleted medium (complete medium depleted of FBS-derived exosomes by overnight centrifugation at 100 000 × *g*) for 24 h. And the culture supernatant was collected. To verify the role of exosomes, GW4869 (10 μM, MCE) was used to treat the BMSCs for 24 h.

As previously described,[17] the supernatant was centrifuged at 4 °C successively at 300 × *g* for 10 minutes, 2000 × *g* for 10 minutes, 10 000 × *g* for 30 minutes, and 100 000 × *g* for 70 minutes. The obtained precipitates were resuspended in 100 μL PBS, and then the total protein in exosome was quantified by a Bradford Protein Assay kit (Beyotime Biotechnology). The characteristics of exosomes were observed under transmission electron microscopy (TEM) and Nanoparticle-tracking analysis (NTA). The protein levels of surface markers (TSG 101, CD81, and CD63) were examined by Western blotting. Exosomes were further characterized by FACS analysis as previously described.[18]

Coculture of Pancreatic Cells With BMSCs In Vitro

Freshly isolated islets were incubated overnight, and then islets (200 IEQ/well) were cocultured with BMSCs (2 × 10^5/

well) in direct contact in 6-well culture plates for 48 h in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin. Moreover, BMSCs were plated into the lower chamber, whereas INS-1 cells were seeded into the transwell plate filters in which the pore is 0.4 μm so that only factors secreted by BMSCs, not BMSCs themselves, can go through the filters in the transwell plates. The cells were then cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, and 50 μM β-mercaptoethanol.

Lentivirus Production and Transfection of INS-1 Cells

Lentiviral clones overexpressing miR-21-5p and the control vector were purchased from GeneChem Company (Shanghai, China). The titer of lentivirus produced was approximately 2×10^8 pfu/mL. Lentivirus transfection of INS-1 cells was performed according to the manufacturer's instructions. After 72 h, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to verify the transfection efficiency. Stable cell lines were then selected by supplementation of the medium with 7.5 μg/mL puromycin.

MiRNA Reagents and Cells Transfection

Mimics NC, miR-21-5p mimics, inhibitor NC, and miR-21-5p inhibitor were purchased from Shanghai Gemma Pharmaceutical Technology (Shanghai, China) and resuspended in DEPC water. The concentration was 20 μM in the experiments.

A 1-μg plasmid pool was transfected into 100 IEQ islets using Lipofectamine-3000 reagent (Invitrogen) as previously described.[19] Transplantation was then performed 24 h after transfection.

To analyze the effect of miR-21-5p on INS-1 cell apoptosis, cells were randomly divided into 4 groups and transfected with Lipo-3000 according to the manufacturer's protocol. The 4 groups were as follows: (a) mimics NC group, INS-1 cells transfected with mimics NC; (b) mimics group, INS-1 cells transfected with specific miR-21-5p mimics; (c) inhibitor NC group, INS-1 cells transfected with inhibitor NC; and (d) inhibitor group, INS-1 cells transfected with the miR-21-5p inhibitor.

Moreover, BMSCs were transfected with these 4 miRNA reagents using Lipo-3000. Twenty-four hours of transfection, the medium was changed to an exosome-depleted medium, and 24 h later, the medium was harvested for exosome isolation. RT-qPCR was used to measure the transfection efficiency.

Apoptosis Analysis by Flow Cytometry and PI/Hoechst Staining

For cell death assays, INS-1 cells were transfected with lentivirus and treated with 3 mM STZ for 24 h as indicated. According to the manufacturer's instructions, the cells were stained with annexin V and 7-AAD (Annexin V Apoptosis Detection Kit I, BD Pharmingen). For each analysis, 10 000 events were recorded. All experiments were run in triplicate. The data were analyzed using FlowJo software v10.

Cell nuclei were stained by Hoechst staining, and cells in different groups were stained with Propidium Iodide to label dead cells as described previously.[20] Images were taken with a Nikon eclipse fluorescence microscope (Nikon, Tokyo, Japan). Data were represented as % of dead cells.

Cell Activity and AO-PI Staining

Cell activity assays were performed using a Cell Counting Kit-8 (CCK8) colorimetric assay (Beyotime Biotechnology), and cells were plated in 96-well plates according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (BioTek, CA, USA).

The islet viability was evaluated after the cells were stained with acridine orange (AO) and propidium iodide (PI) (Sigma-Aldrich) as described.[21]

Glucose-Stimulated Insulin Secretion (GSIS) In Vitro

One hundred IEQ islets were seeded into each well; thus, the concentrations of insulin in the supernatants were directly used for the evaluation of insulin secretion. The islets were cultured in the presence of low (2.8 mM) and high concentrations of glucose (16.7 mM) for 2 h. The medium was collected twice, and the insulin levels were determined using the Rat Insulin ELISA Kit (Mercodia).

RNA Preparation and RT-qPCR

RNA samples were prepared and analyzed by RT-qPCR as described previously. Briefly, cells were harvested, prepared using TRIzol reagent (Invitrogen), and then analyzed by RT-qPCR. For miRNA analysis, cDNAs were generated and amplified using the Mir-X miRNA First-Strand Synthesis Kit (Takara). U6 was used as a reference for miRNA analysis.

A high-capacity cDNA reverse transcription kit (Roche) was used to reverse-transcribe 1 μg of total RNA into cDNA. Quantitative PCR (qPCR) was performed using quantitative SYBR Green PCR Master Mix (Qiagen) and an ABI 7500 Fast Real-Time (RT) PCR system (Thermo Fisher Scientific) according to the manufacturer's cycling conditions. The housekeeping gene GAPDH was used as an internal reference gene.

The primers for rat Bcl-2, Bax, PDCD4, Insulin, and GAPDH were synthesized by Tsingke Biotechnology Company (Peking, China) and are shown in Supplementary Table S1.

RNA Sequencing

The gene expression profiles of subjects in the miR-21-5p overexpression group and the control group were compared using an Illumina Novaseq 6000, and the whole transcriptome was examined by Lianchuan Biotechnology (Hangzhou, China).

Data normalization and differential expression analysis were performed using the R package DESeq2, v. 1.16.1. Differentially expressed genes (DEGs) were filtered using the following parameters: $P < .05$ and a filtering threshold of fold change (FC) > 1.5. Volcano plots and heat maps of these screened DEGs were then constructed using R software.

Western Blotting Analysis

The treated cells were collected and lysed with RIPA lysis buffer (Beyotime). The protein concentration was determined using a BCA protein assay kit (Beyotime). Cell lysates (20 μg) were separated by a 12.5% SDS-PAGE gel, and the proteins were then transferred to a PVDF membrane. The membrane was blocked with 5% bovine serum albumin (BSA) for 1 h and then incubated with the indicated primary antibodies at 4 °C overnight. The membranes were washed and incubated with secondary antibodies (1:5000) for 1 h at room temperature. An enhanced chemiluminescence Western blotting detection kit (Bio-Rad) was used to produce signals for imaging. The optical density of each band was analyzed using ImageJ software (National Institutes of Health [NIH]).

The primary antibodies used were mouse anti-Bcl-2 (1:1000, Cell Signaling Technology, #15071S), rabbit

anti-Bax (1:1000, Cell Signaling Technology, #2772S), rabbit anti-Caspase3 (1:1000, Cell Signaling Technology, # 9662S), rabbit anti-PDCD4 (1:1000, Cell Signaling Technology, # 9535S), mouse anti-TSG 101 (1:500, Santa Cruz Biotechnology, sc-7964), mouse anti-CD81 (1:500, Santa Cruz Biotechnology, sc-166029), mouse anti-CD63 (1:500, Santa Cruz Biotechnology, sc-365604), and mouse anti-GAPDH (1:5000, Proteintech, 60004-1-Ig).

Dual Luciferase Assay

INS-1 cells were plated on 12-well plates (10 000 cells/well) overnight and cotransfected with 2 μg of the constructed plasmid together with miR-21-5p mimics using 5 μL of Lipo-3000 reagent according to the manufacturer's protocol. Forty-eight hours after transfection, luciferase activity was measured using the Dual Luciferase assay system (Promega), as previously described.[22]

Islet Transplantation

Male Lewis rats (250-300 g) were used as transplant donors, and male Lewis rats (100-150 g) were used as recipients. Islets were transplanted under the renal capsules of the recipient rats as described previously. Briefly, islets were loaded in a polyethylene PE50 tube (Becton Dickinson) and injected using a Hamilton syringe.

The diabetes model was established in rats weighing 100-150 g through an intraperitoneal injection of 55 mg/kg streptozotocin (STZ, Sigma-Aldrich), and the rats were considered diabetic when the nonfasting blood glucose concentration exceeded 16.7 mmol/L for more than 3 consecutive days. Seven days after STZ injection, the diabetic rat model was used for subsequent experiments. The blood glucose concentrations were measured using a blood glucose meter (Novo Net). The diabetic rats were randomly divided into 3 groups: (a) nontransplant group (RPMI 1640, n = 12); (b) islets-mimics NC group (2000 islet equivalents [IEQ], n = 12); and (c) islets-mimics group (2000 islet equivalents [IEQ], n = 12). The nonfasting blood glucose level and weight were measured on postoperative days (PODs) 0, 3, 5, 7, and 14. On POD 7 and 14, 6 animals in each group were randomly selected, and the right kidney was removed.

Intraperitoneal Glucose Tolerance Test (IPGTT) In Vivo

For the IPGTT, mice were fasted for 6 h and intraperitoneally injected with D-glucose (2 g/kg) (Sigma-Aldrich). Blood samples were collected at 0, 15, 30, 60, 90, and 120 minutes after glucose injection from the tail vein, and glucose levels were analyzed using a glucometer.

TUNEL Assay

TUNEL assays were performed using a TUNEL assay kit (Beyotime Biotechnology) according to the manufacturer's instructions. Fluorescent photographs of cells were captured by a fluorescence microscope (Zeiss). To determine the apoptotic rate, 5 visual fields were selected randomly from each section, and the numbers of positive cells and total cells were determined with ImageJ. The percentage of TUNEL-positive cells/total cells was calculated for statistical analysis.

Immunohistochemical (IHC) Analysis of Grafted Islets

Kidneys bearing grafts were removed at the indicated time points for IHC analysis. The tissues were fixed with 4% paraformaldehyde, and then the grafts were directly embedded in paraffin, whereas the cells were fixed in agarose gel, dehydrated with an ethanol gradient and washed with xylol, and embedded in paraffin. The samples were sliced into 4-μm-thick sections.

The slides were subjected to IHC analysis using Rabbit anti-insulin antibody (1:700; Servicebio, GB11334). The slides were imaged with a Nikon microscope system.

Immunofluorescence (IF) Staining

IF staining was performed as previously described.[23] Images were taken using a Leica DMR HC epifluorescence microscope (Leica Microsystems). To analyze the relationship between miR-21-5p and PDCD4, we immunofluorescently double-stained the sections for Insulin (1:500; Abcam) and PDCD4 (1:3000; Cell Signaling Technology) and analyzed a minimum of 100 IEQ islets per section. Positively stained cells and the area of the fluorescence signal were used for quantification with ImageJ analytical software.

Statistical Analysis

The statistical analyses were performed using SPSS 19.0 software (SPSS Inc.) and GraphPad Prism 9.0 (GraphPad Inc.). All data are presented as the means ± standard deviation (SD). The significance was tested using Student's t test. A value of $P < .05$ was considered to indicate significance.

Results

Isolation and Identification of Islets, BMSCs and BMSCs-Derived Exosomes

The whole bone marrow adherence method was used to isolate and culture MSCs from rats.[24] As observed under a light microscope, passage 3 BMSCs were typical spindle-shaped cells (Supplementary Fig. S1A). Flow cytometry was performed to characterize BMSC surface markers, and these cells were positive for CD90 and CD29 and negative for CD45 and CD11b/c (Supplementary Fig. S1B). Oil Red O and alizarin red staining showed that BMSCs successfully underwent adipogenic and osteogenic induction, indicating that BMSCs had multilineage differentiation ability (Supplementary Fig. S1C). Density gradient centrifugation was performed for the separation of islets, and we then used DTZ to identify islets by selectively staining β-cells red (Supplementary Fig. S1D). These results indicated that BMSCs and islets were successfully isolated and cultured, and subsequent experiments were performed with these cells.

We performed differential centrifugation to isolate exosomes from the culture supernatants of BMSCs. NTA and TEM were performed to identify the shape and size of the exosomes (Supplementary Fig. S1E, F). Further Western blotting analysis then revealed that the exosomal marker proteins TSG101, CD81, and CD63 were enriched in exosomes (Supplementary Fig. S1G). Taken together, these results show that we successfully isolated high-purity exosomes from BMSCs.

BMSCs and BMSCs-Derived Exosomes Protect Islets and INS-1 Cells Against Apoptosis

To evaluate apoptosis in islets and INS-1 cells after 48 h of coculture with BMSCs, we performed Western Blotting to measure Bcl-2, Bax, and cleaved Caspase-3 levels. In pancreatic islets and INS-1 cells that were cocultured with BMSCs, Bcl-2/Bax ratios were increased, which were consistent with the changes in mRNA levels (Supplementary Fig. S5A, B), and cleaved Caspase-3 expression levels were significantly decreased (Fig. 1A). Our previous study also confirmed that

cotransplantation of islets and BMSCs could significantly reduce the expression level of cleaved Caspase-3 and improve the activity of transplanted islets.[8]

Growing evidence supports that stem cells improve islet transplantation outcomes via paracrine mechanisms and exosomes play important roles.[25,26] To determine whether BMSCs-derived exosomes could reduce β-cell apoptosis, we first treated cells with different concentrations of exosomes (0, 6.25, 12.5, 25, 50, and 100 μg/mL) for 48 h (Supplementary Fig. S2). Moreover, treatment with 50 μg/mL BMSCs-Exo markedly increased the Bcl-2/Bax ratios and decreased the cleaved Caspase-3 expression levels both in islets and INS-1 cells (Fig. 1B and Supplementary Fig. S5C, D). Collectively, these findings indicate that BMSCs and BMSCs-Exo protect islets and INS-1 cells against apoptosis.

BMSCs Reduce Apoptosis of Pancreatic Cells by Delivering Exosomal miR-21-5p

Studies have found that exosomes are rich in miRNA, we then consulted the database of miRNAs secreted by BMSCs and selected the top 5 miRNAs with the most abundant expression according to the 2 reviews[27,28] on miRNAs and islet function (Fig. 1C). Previous studies showed that miR-15b-5p plays an essential role in insulin secretion during the development of diabetes,[29,30] so we selected miR-21-5p as the research object. The RT-qPCR results showed that miR-21-5p mRNA levels were significantly upregulated 1.5-fold and 4.8-fold in pancreatic islets and INS-1 cells that were cocultured with BMSCs (Fig. 1D, E), respectively.

To explore whether BMSCs upregulated miR-21-5p expression in INS-1 cells by secreting exosomes and transferring miR-21-5p, cells were treated with GW4869, a neutral sphingomyelinase inhibitor that inhibits exosome secretion, for 24 h before being cocultured with BMSCs. We found that the expression level of miR-21-5p in islets and INS-1 cells significantly decreased 0.5-fold (Fig. 1D, E). Furthermore, miR-21-5p levels were significantly upregulated both in islets and INS-1 cells after exposure to BMSCs-Exo (Fig. 1F, G). Additionally, as shown in Fig. 2A, exosomes can be uptaken by INS-1 cells. Next, we transfected BMSCs with miR-21-5p

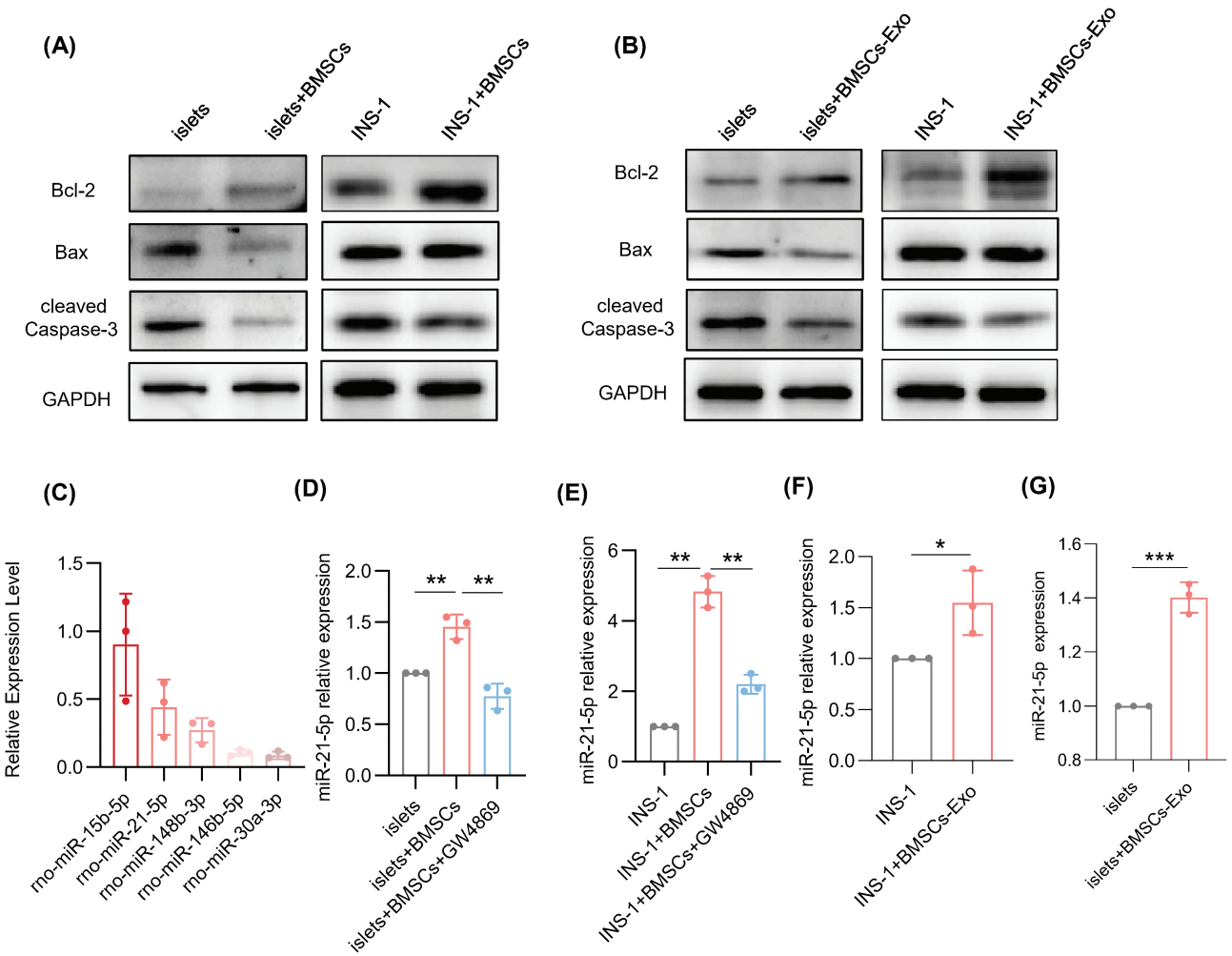

Figure 1. BMSCs and BMSCs-derived exosomes decrease apoptotic cell death in islets and INS-1 cells. **A**, Western blotting analysis of Bcl-2, Bax, and cleaved Caspase-3 in islets (left) and INS-1 cells (right) cultured alone or cocultured with BMSCs for 48 h. **B**, These apoptosis related proteins were detected by Western blotting analysis in islets (left) and INS-1 cells (right) following exposure BMSCs-Exo. **C**, BMSCs exosomes contained abundant miRNAs. MiR-15b-5p, miR-21-5p, miR-148b-3p, miR-146b-3p, and miR-30a-3p were the top 5 most abundant miRNAs in BMSCs-derived exosomes. RT-qPCR analysis of the expression of miR-21-5p in islets (**D**) and INS-1 cells (**E**) after coculture and treated with or without GW4869. **F** and **G**, miR-21-5p expression levels in islets and INS-1 cells following exposure BMSCs-Exo. **P* < .05, ***P* < .01, and ****P* < .001; *n* = 3. Data are represented as mean ± SD.

mimics NC, mimics, inhibitor NC and inhibitor, respectively (Fig. 2B), and then isolated exosomes derived from these 4 groups. RT-qPCR results confirmed the transfection efficiency (Fig. 2C). Moreover, miR-21-5p expression level in INS-1 cells was upregulated following coculture with BMSCs-mimics-derived exosomes ($BMSCs^{mimics}$-Exo) and downregulated following coculture with BMSCs-inhibitor-derived exosomes ($BMSCs^{inhibitor}$-Exo) (Fig. 2D). In summary, these results demonstrate that miR-21-5p could enter pancreatic cells through exosomes.

Subsequently, we studied whether the protective effect of BMSCs-Exo on β-cells might be attributed to miR-21-5p. Streptozocin (STZ) was used to simulate early engrafted islet ischemia and hypoxia in vitro.[31] Our previous study[20] indicated that 3 mM STZ was a suitable concentration for treatment of INS-1 cells, and our RT-qPCR results also demonstrated that the Bcl-2/Bax mRNA ratio was significantly decreased (Supplementary Fig. S5E). As expected, PI/Hoechst staining results showed that apoptosis declined significantly in INS-1 cells following exposure to $BMSCs^{mimics}$-Exo compared to $BMSCs^{mimics\ NC}$-Exo (Fig. 2E, F). However, when miR-21-5p expression level was inhibited in BMSCs-Exo, the apoptosis of INS-1 cells was increased (Fig. 2E, F). The results of Western blotting and RT-qPCR showed the same results, as shown in Fig. 2G and Supplementary Fig. S5F, G. Thus, our data demonstrate that miR-21-5p seems to play a significant role in BMSCs-derived exosomes alleviated apoptosis in β-cells.

MiR-21-5p Alleviates Apoptosis in INS-1 Cells

To verify the effect of miR-21-5p on INS-1 cells, we transfected the cells with a miR-21-5p lentivirus (Fig. 3A) and examined apoptosis. Using the CCK8 assay, we found that the survival rate of INS-1 cells decreased by 40% in response to 3 mM STZ for 24 h (Fig. 3B), and cell viability was significantly increased when miR-21-5p expression was increased. Flow cytometry analysis was then used to further verify this result. The percentage of apoptotic cells was approximately 19.3% among miR-21-5p-overexpressing INS-1 cells treated with 3 mM STZ, while the apoptosis rate was approximately 6.5% in control cells (Fig. 3C, D). Moreover, we measured the levels of apoptosis-related factors in miR-21-5p overexpressing and NC cells and found that Bcl-2/Bax protein ratio was increased significantly, while cleaved Caspase-3 expression level was decreased (Fig. 3E). The RT-qPCR results revealed that miR-21-5p overexpression also reduced apoptosis in response to 3 mM STZ (Supplementary Fig. S5I).

To further explore the relationship between miR-21-5p and apoptosis, miR-21-5p mimics, a control mimic, miR-21-5p inhibitor, and a control inhibitor were individually transfected into INS-1 cells. After transfection, miR-21-5p expression in mimics transfected cells was markedly elevated, while that in inhibitor transfected cells was substantially decreased compared to that in cells transfected with their respective controls (Fig. 3F). In addition, compared with that in the mimics NC group, the Western blotting results showed a significantly increased Bcl-2/Bax ratio and downregulated cleaved Caspase-3 expression in the miR-21-5p mimics group. In contrast, the miR-21-5p inhibitor group showed that the ratio of Bcl-2/Bax was reduced, and markedly upregulated cleaved Caspase-3 expression level (Fig. 3G). Furthermore, these Western blotting results were consistent with the RT-qPCR results (Supplementary Fig. S5J, K). Meanwhile, as shown in Fig. 3H and I, the PI/Hoechst staining results suggested that treatment with 3 mM STZ for 24 h resulted in a 9.3% cell death in the mimics group compared to the mimics NC group (23.0%), and a 43.0% cell death in the inhibitor group versus the inhibitor NC group (24.3%). Collectively, these results show that miR-21-5p overexpression significantly reduced INS-1-cell apoptosis.

MiR-21-5p Enhances Insulin Secretion by Pancreatic β-Cells and Protects Cells From Apoptosis

After successfully transfecting mimics into islets using Lipo-3000 (Fig. 4A), We performed AO-PI staining to distinguish living cells from dead cells in the islet mass. As expected, the survival rate of islet cells transfected with the mimics was 1.5 times higher than that of the control group (Fig. 4B, C). Subsequently, we examined Bcl-2, Bax, and cleaved Caspase-3 expression levels. As shown in Fig. 4D and Supplementary Fig. S5L, the changes in these apoptosis-related factors were consistent with those in INS-1 cells. Furthermore, to examine the effects of miR-21-5p on islet survival, we evaluated β-cell function through assays of insulin release. The results showed that islets that were transfected with mimics released 1.4-times more insulin than those in the control group (Fig. 4E). Additionally, the RT-PCR results indicated that miR-21-5p may increase the expression of insulin mRNA (Fig. 4F). Taken together, these results indicate that miR-21-5p could reduce islet β-cell apoptosis and improve the islet function.

MiR-21-5p Targets PDCD4

To gain insights into the mechanism through which miR-21-5p mediates apoptosis in INS-1 cells, we examined miR-21-5p target genes using genome-wide transcriptomics. Whole-transcriptome (RNA) sequencing (Supplementary Fig. S3A) was performed on stable miR-21-5p-overexpressing and negative control cells. We analyzed the genes associated with miR-21-5p overexpression, and the results indicated that 708 genes showed significantly differential expression between INS-1-NC and INS-1-miR-21-5p cells. The total number of DEGs was 708, which included 521 upregulated and 187 downregulated DEGs (Supplementary Fig. S3B). The volcano plot of DEGs (Supplementary Fig. S3C) showed that 73.59% of all DEGs were upregulated and 26.41% were downregulated. We used miRecords bioinformatics software, miRWalk, TargetScan and miRTarBase to forecast possible miR-21-5p target genes, and the tumor suppressor gene PDCD4 was identified in the 4 databases (Fig. 5A). As shown in the heatmap (Fig. 5B), PDCD4 transcription was significantly downregulated in INS-1 cells after miR-21-5p overexpression.

In INS-1 cells transfected with miR-21-5p lentivirus and treated with miR-21-5p mimics, the mRNA and protein expression levels of PDCD4 were significantly decreased (Fig. 5C-F). However, when we used a miR-21-5p inhibitor, the RT-qPCR and Western blotting results showed markedly upregulated expression of PDCD4 (Fig. 5E, F). In islets, PDCD4 was significantly decreased with the increase in miR-21-5p expression (Fig. 5G, H). These data suggested that the upregulation of miR-21-5p was associated with a decrease in PDCD4 mRNA expression.

To explore the relationship between miR-21-5p and PDCD4, we performed a dual luciferase reporter gene

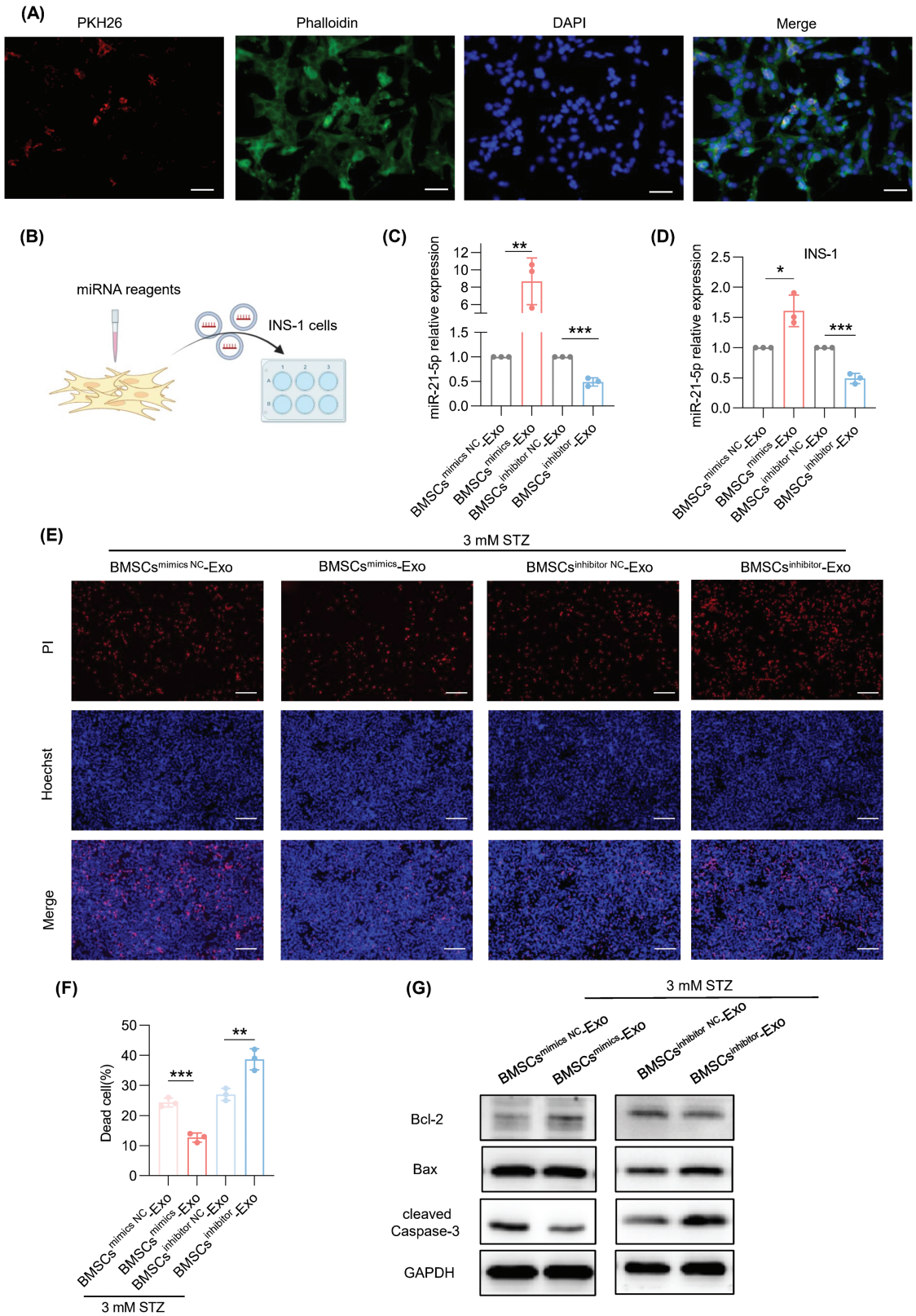

Figure 2. BMSCs reduce apoptosis of pancreatic cells by delivering exosomal miR-21-5p. **A**. Exosomes were uptaken by INS-1 cells. Cells were stained with a phalloidin conjugate (green), nuclear counterstaining was performed using DAPI (blue), nanovesicles were labeled with PKH26 (red). Scale bars: 50 μm. **B**, Schematic diagram of BMSCs transfected with miRNA reagents, and extract the exosomes to treat INS-1 cells. The expression levels of miR-21-5p in BMSCs-derived exosomes (**C**) and INS-1 cells (**D**) were detected by RT-qPCR in different groups. **E** and **F**, The PI/Hoechst staining for apoptosis in INS-1 cells after cocultured with different BMSCs-derived exosomes under 3 mM STZ treatment for 24 h. Scale bars: 100 μm. G, Apoptosis related proteins were detected by Western Blotting. *$P < .05$, **$P < .01$ and ***$P < .001$; $n = 3$. Data are represented as mean ± SD.

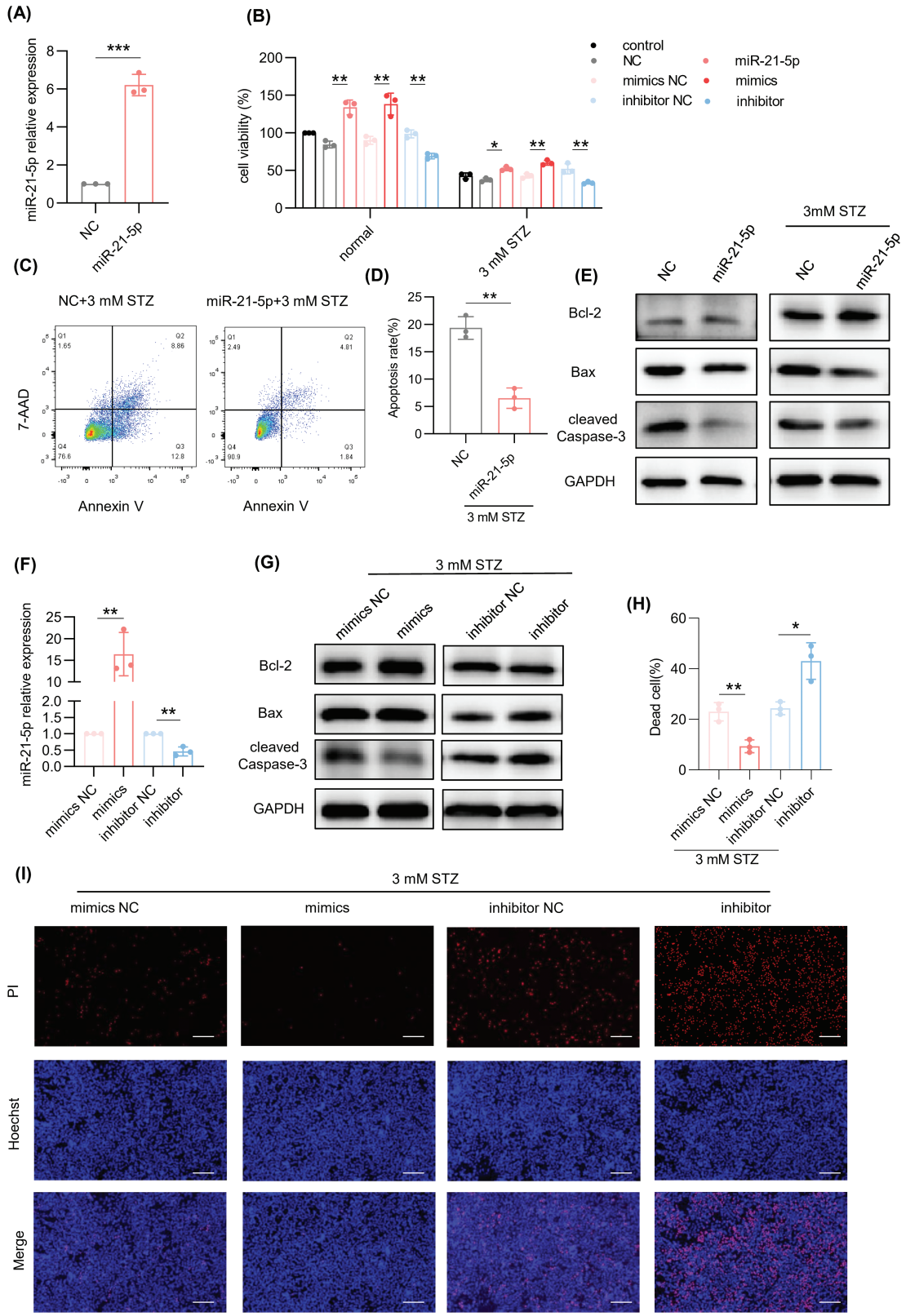

Figure 3. MiR-21-5p suppresses apoptosis in INS-1 cells. A and F, The transfection efficiency was observed by RT-qPCR following transfection with miR-21-5p lentivirus, mimics, and inhibitor at 48 h. B, Cell viability was measured by CCK8 analysis. C and D, MiR-21-5p overexpressing or NC cells were exposed to 3 mM STZ for 24 hours, and cell apoptosis was measured by flow cytometry analysis with Annexin V/7-AAD staining. E and G, MiR-21-5p affects apoptotic protein levels. Cells in different groups were analyzed by Western blot analysis to determine the levels of Bcl-2, Bax, and cleaved Caspase-3. H and I, INS-1 cells in mimics NC, mimics, inhibitor NC and inhibitor groups were stained with Hochest33342 and PI, imaged and the percentages of apoptotic cells were counted. Scale bars: 100 μm. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$; n = 3. Data are represented as mean ± SD.

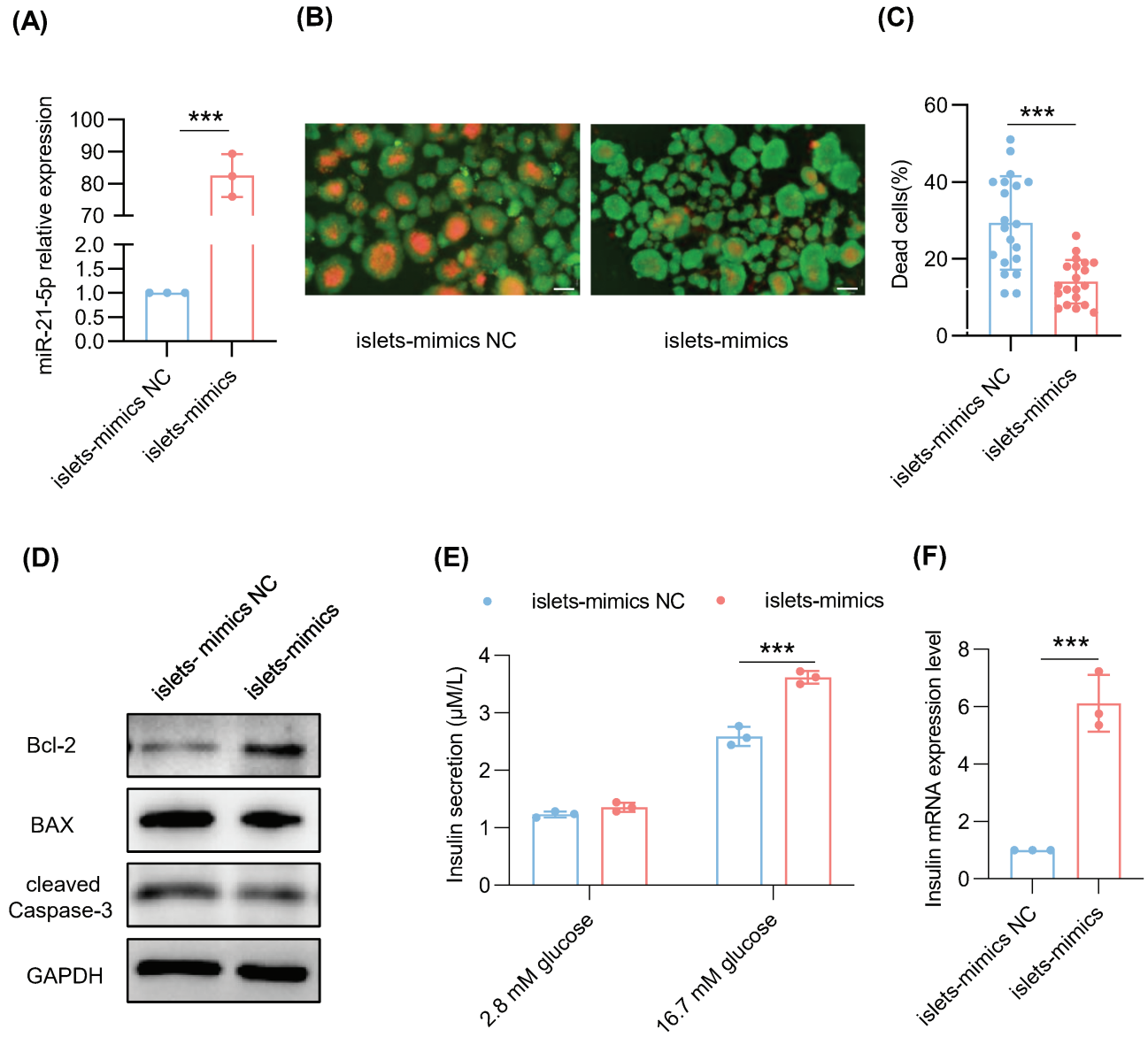

Figure 4. MiR-21-5p improves islet viability and alleviates apoptosis in pancreatic islets. **A**, Quantitative analysis of the transfection efficiency in islets. **B** and **C**, AO-PI double-fluorescent staining was performed for the visualization of islet viability. Viable islets are green and dead islets are red. Scale bars: 100 μm. **D**, Western blotting analysis for islets and apoptosis markers (Bcl-2, Bax, and cleaved Caspase-3,) were shown. **E**, GSIS assays were performed in islets transfected with mimics NC and mimics. **F**, The results from the RT–qPCR analysis of insulin were shown. *$P < .05$, **$P < .01$, ***$P < .001$; $n = 3$. Data are represented as mean ± SD.

experiment (Fig. 5I). The results revealed that miR-21-5p overexpression considerably reduced the luciferase activity of the PDCD4-WT luciferase reporter vector compared with the results found for the negative control. In contrast, miR-21-5p overexpression did not affect the luciferase activity of PDCD4-MUT (Fig. 5J). This finding indicated that the transcriptional regulation of miR-21-5p and PDCD4 occurred in a direct manner. These results suggest that the inhibitory effect of miR-21-5p on apoptosis might be mediated in part by targeting PDCD4.

MiR-21-5p Overexpression in Pancreatic Islets Improves the Function of Grafted Islets and Decreases Apoptosis In Vivo

Diabetes was induced in adult male Lewis rats by a single STZ injection.[32] We then selected rats with 3 consecutive blood glucose measurements greater than 16.7 mM/L for our subsequent experiments. To determine the effects of miR-21-5p transfection on islet grafts, we designed a rat experiment (Fig. 6A).

We compared the effect of miR-21-5p mimics transfection of islet grafts on STZ-induced diabetic rats with the effects of the mimics negative control. Nonfasting blood glucose and body weight were monitored for 14 days post-transplantation. As shown in Fig. 6B, a significant decrease in blood glucose levels was detected after islet transplantation. On POD 5, the level in the mimics group decreased significantly compared to that in the mimics NC group ($P < .05$). Beginning on day 7 after transplantation, the difference in the blood glucose levels between the 2 groups was even more significant ($P < .01$). However, there was no significant difference in body weight among the 3 groups in our study (Fig. 6C).

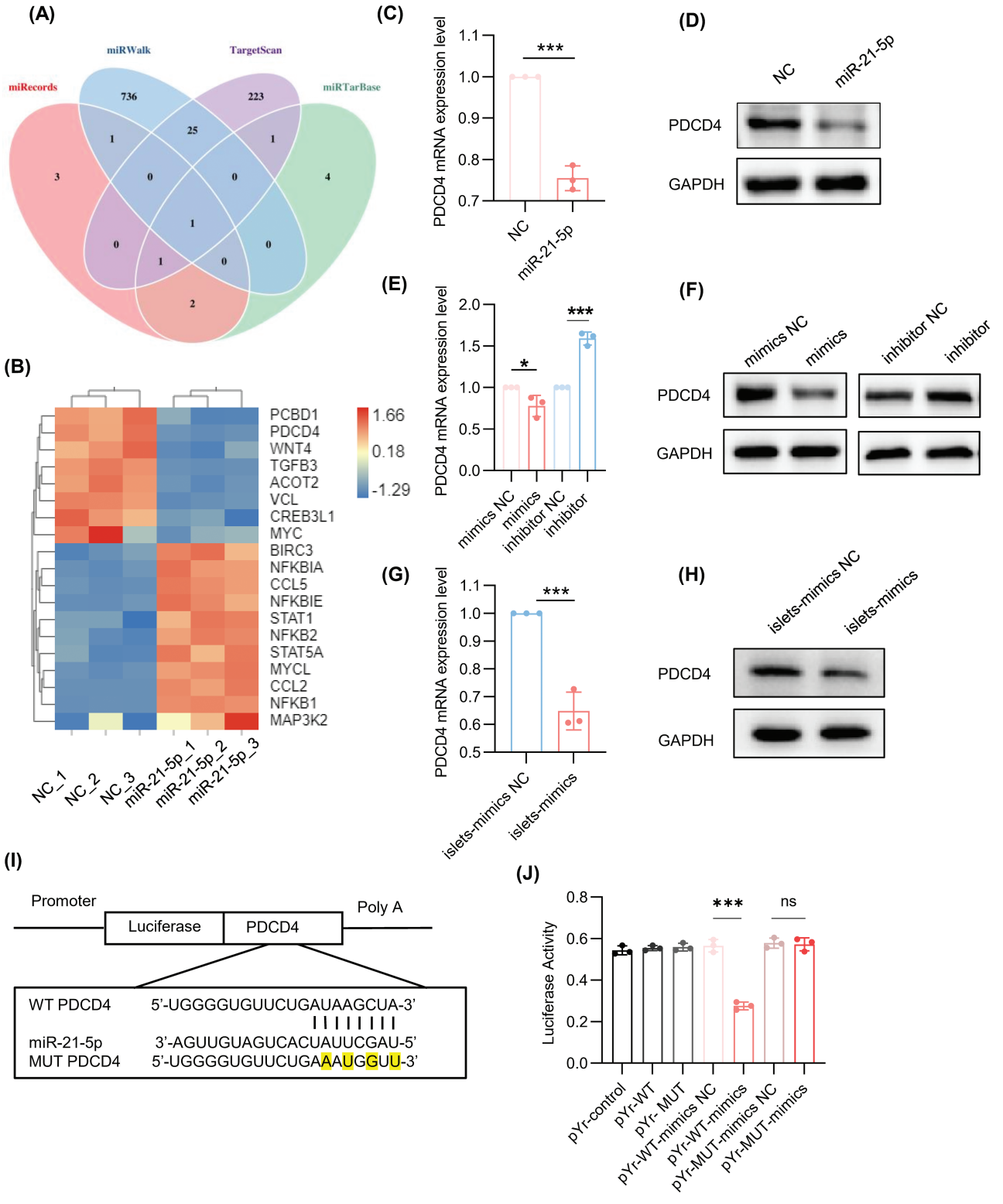

Figure 5. MiR-21-5p targets PDCD4. **A**, The 4-way Venn diagram showing miR-21-5p target genes identified by miRecords, miRWalk, TargetScan, and miRTarBase. **B**, Heatmap representation of the transcriptome results of the top 10 upregulated or downregulated genes. **C** and **D**, Expression of PDCD4 in INS-1 cells transfected with lentivirus, as determined by RT-PCR and Western blotting. NS-1 cells were successfully transfected with miR-21-5p mimics NC, mimics, inhibitor NC and inhibitor, and then the PDCD4 expression level was measured at mRNA (**E**) and protein (**F**) levels, respectively. **G** and **H**, RT-PCR and Western blotting analysis of PDCD4 expression in islets after transfection with miR-21-5p mimics NC and mimics for 24 h. **I**, The predicted binding sequences of miR-21-5p in the wild-type and mutant targeting regions of PDCD4. J, INS-1 cells were double transfected with miR-21 mimics NC or mimics and PDCD4_WT or PDCD4_MUT. The dual luciferase assays were then performed, and relative luciferase activities were measured. **P* < .05, ***P* < .01, ****P* < .001; ns, not significant; *n* = 3. Data are represented as mean ± SD.

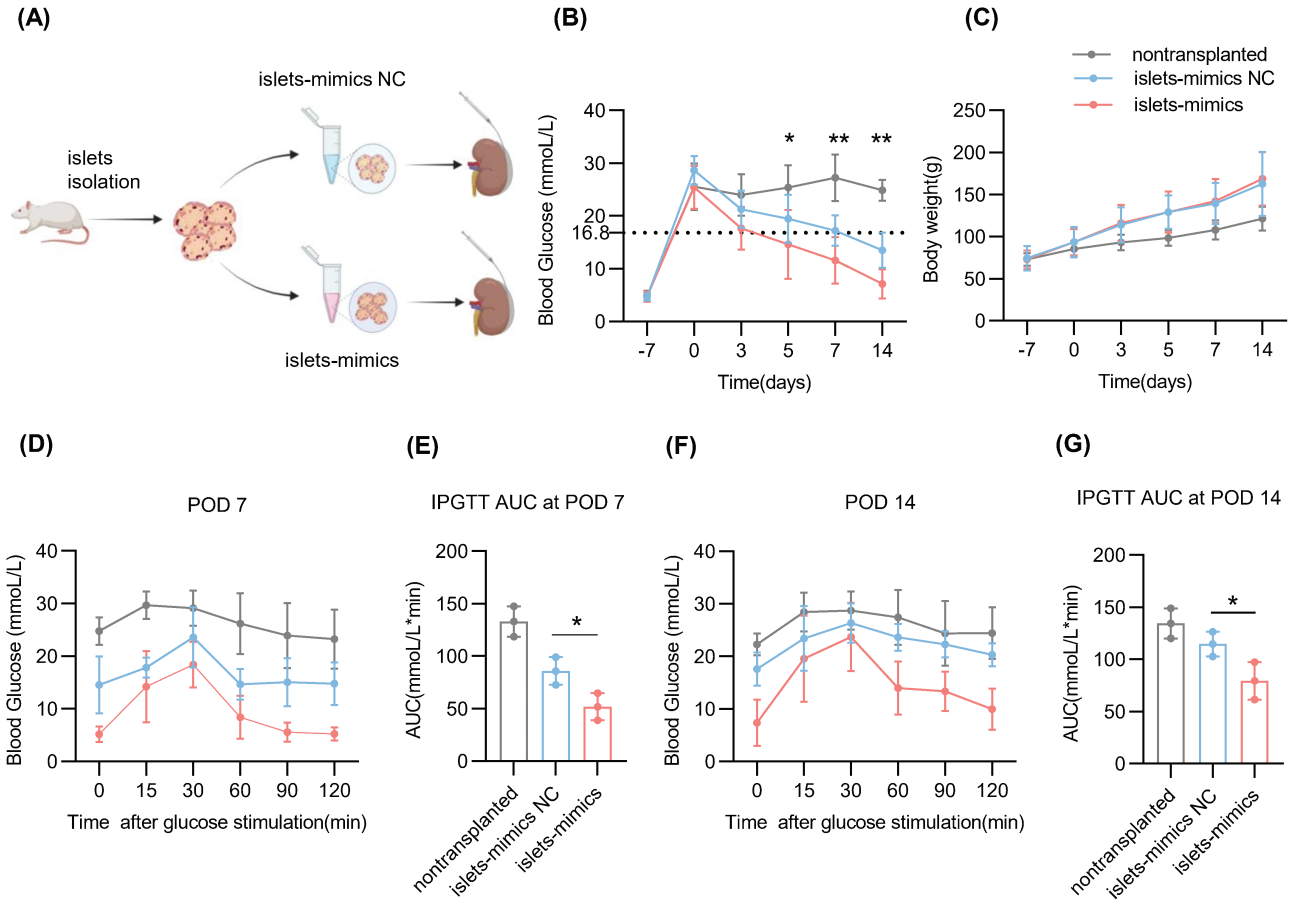

Figure 6. Transplantation of miR-21-5p overexpressing islets significantly accelerates the normalization of blood glucose in diabetic rats. **A**, Grouping diagram of islet transplantation. **B** and **C**, Blood glucose and body weight changes in nontransplanted, mimics NC and mimics groups. IPGTT (2 g/kg) was performed at 7 (D) and 14 (F) days after transplantation in the 3 groups. **E** and **G**, Quantification of the area under curve. *$P < .05$, **$P < .01$; $n = 6$. Data are represented as mean ± SD.

To further evaluate the islet engraftment efficacy, we performed glucose tolerance tests at 7 d and 14 d after transplantation. The area under the curve (AUC) for the blood glucose levels was significantly reduced in all islet transplantation groups, and the miR-21-5p mimics islets showed improved glucose tolerance compared to transplanted mimics NC islets (Fig. 6D-G).

The animals were sacrificed on POD 7 and 14, and the secretion of insulin by islet cells after transplantation was observed by immunohistochemical staining (Fig. 7A, B), in which the isotype IgG was used as negative control (Supplementary Fig. S4A). The administration of miR-21-5p mimics under the renal capsule on day 7 after transplantation resulted in a 3.2-fold (vs. mimics NC) increase in the insulin-positive area. To confirm apoptosis, the grafts were examined by TUNEL staining. Consistent with our in vitro results, the imaging results showed that the apoptosis rate was significantly reduced in the mimics group compared with the mimics NC group (Fig. 7C, D). We also used a PDCD4/INSULIN dual immunofluorescence staining approach to examine the expression level of PDCD4 in grafted islets. Results indicated that the percentage of PDCD4^{+}/ INSULIN^{+} cells was markedly lower in the mimics group than in the mimics NC group (Fig. 7E, F). These results suggest that miR-21-5p could ameliorate the impairment in glucose tolerance and insulin secretion and significantly reduce apoptosis by targeting PDCD4.

Discussion

Pancreatic islet transplantation is considered a practical therapeutic approach for T1DM[33]; however, in the early posttransplantation period, hypoxia and nonspecific inflammatory events cause significant β-cell loss and early islet graft dysfunction.[34-36] Results by Montserrat Biarnes et al have shown that on day 3 after transplantation, β-cell apoptosis was 10 times higher than that in the normal pancreas.[37] Therefore, it is essential to maximally reduce the islet loss during the early posttransplantation period.[38] The data collected in this study provide a new strategy to reduce the apoptosis of early transplanted islets and improve islet function.

Most previous studies provide compelling evidence showing that MSCs can significantly improve the efficacy of islet transplantation in various ways, such as by promoting islet function and inhibiting immune rejection.[39-41] However, the underlying molecular mechanisms remain largely unclear. Due to potential risk factors,[42] such as ethical issues and immune responses, many challenges need to be addressed before the clinical application of MSCs. Therefore, direct MSC transplantation may not be an optimal choice. Exosomes contain abundant bioactive molecules from stem cells and have fewer potential risks, and exosomes thus offer a safe, biomimetic alternative with lower oncogenic and immunological risks and greater target specificity than MSCs.[43] Exosomes are well known to play vital roles in cell-to-cell communications by carrying many types of

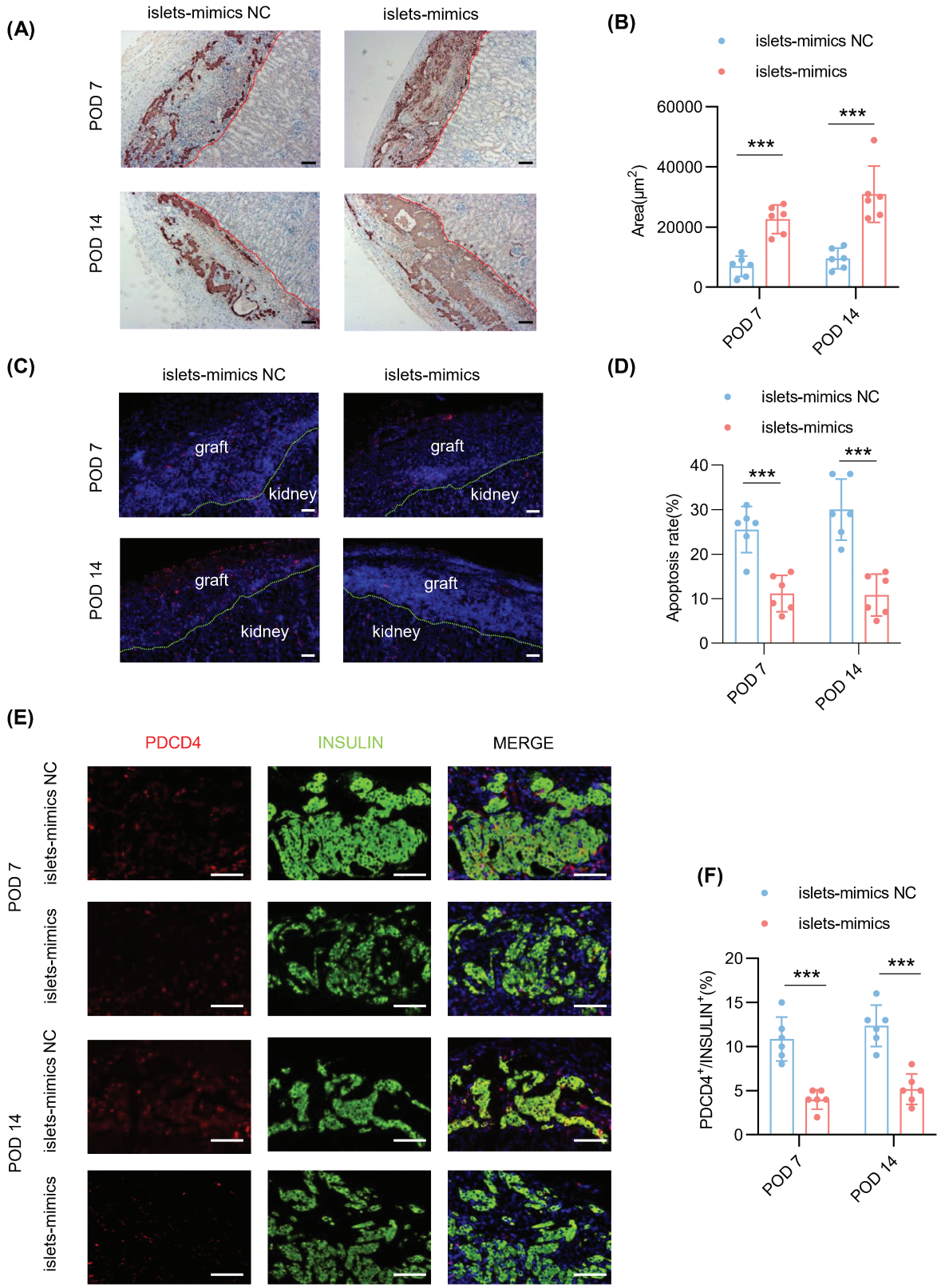

Figure 7. MiR-21-5p overexpression in islets protects islets and against apoptosis. **A** and **B**, Typical images and quantitative results of insulin immunohistochemistry in the mimics NC and mimics groups on POD 7 and 14. **C** and **D**, TUNEL staining of the grafts on POD 7 and 14 and quantification of total TUNEL⁺ nuclei. **E** and **F**, Representative images of IF staining of PDCD4 (red) and INSULIN (green) in grafts of the 2 groups. Scale bars: 100 μm. ***$P < .001$; $n = 6$. Data are represented as mean ± SD.

bioactive molecules, such as proteins, lipids, long noncoding RNAs, and miRNAs.[18,44] MiR-21-5p has been reported to be the most abundant miRNA in MSC-derived exosomes according to miRNA sequencing.[18] Previous studies have demonstrated that the miR-21-5p expression levels in β-cells are significantly elevated in response to cytokines and inflammation, which suggest that miR-21 also plays an essential role in β-cell survival.[45,46] Our results showed that miR-21-5p was overexpressed in islets cocultured with BMSCs and was associated with apoptosis. The aim of the present study was to investigate the potential role of miR-21-5p in islets apoptosis after coculture with BMSCs and elucidate the underlying mechanism.

PDCD4 is one of the most widely researched miR-21-5p target genes. At present, a large number of studies have confirmed that PDCD4 is related to apoptosis. Consistent with the results observed in other cell types, β-cell miR-21-5p has been demonstrated to target the tumor suppressor PDCD4. Qingguo Ruan[47] showed that the NF-κB-miR-21-PDCD4 axis plays a crucial role in pancreatic β-cell death. In our study, we also confirmed these previous results. In the present study, we first showed that miR-21-5p plays an essential role in BMSCs by reducing islets apoptosis. Second, the upregulation of miR-21-5p expression levels in INS-1 cells and islets significantly decreased apoptosis, while the downregulation of miR-21-5p increased the apoptosis sensitivity of cells. Moreover, we used bioinformatics technology to predict the target gene of miR-21-5p and combined these findings with RNA-seq data to identify PDCD4. PDCD4 has been suggested to be a protein that is expressed during apoptosis. A double-luciferase assay was performed to verify this hypothesis. Moreover, we not only proved the protective effect of miR-21-5p in vitro but also creatively transplanted islets transfected with mimics into the STZ-induced diabetic rats to evaluate the protective effect of miR-21-5p in the early stage of transplantation in vivo. Our animal experiments further confirmed our hypothesis that transplanted the islets transfection of miR-21-5p mimics into diabetic rats showed better outcomes than those in the negative control group.

However, data by Sims[48] revealed that miR-21 overexpression could lead to an apparent increase in β-cell death. The differences between our study and previously published work may have several possible explanations but the most likely reason is that INS-1 cells were treated differently. In Sims's study, cells were treated with a cytokine mix consisting of IL-1β, IFN-γ, and TNF-α, while our INS-1 cells were cultured in medium containing 3 mM STZ.

Furthermore, our in vivo and in vitro experimental data revealed that miR-21-5p overexpression improved the insulin release rate. In addition to alleviating apoptosis, there are other possible causes, such as ferroptosis and other forms of programmed cell death, such as the insulin secretion process. Our subsequent research will further explore the mechanism by which miR-21-5p regulates insulin secretion and confirm the target. We also found that vascular endothelial growth factor A (VEGFA) expression level was significantly increased both in cells and grafts, and immunofluorescence staining results of CD34 further indicated that miR-21-5p could also improve the early angiogenesis of grafted islets. A study by Valia Bravo-Egana et al suggested that miR-21 targets PCLO resulting in the impairment of β-cell function and survival in the inflammatory process.[45] Besides, miR-21 may play a role in the allograft rejection process, and silencing of miR-21 leads to prolonged islet allograft survival in mice.[49] Hence, to test the potential role of miR-21-5p in transplanted islets, lymphocytic infiltration and inflammatory cytokines will also be examined in subsequent experiments. Overall, our results suggest that miR-21-5p may play a role in islet transplantation by preventing apoptosis in the early posttransplantation period. In addition, bioinformatics analysis confirmed that PDCD4 was directly regulated by miR-21-5p. However, further research is needed to determine how miR-21-5p can be more safely delivered into islet cells. Fortunately, targeted drug delivery by exosome engineering using genetic and chemical methods have achieved great success.[50] Moreover, nanocarriers[51] and small-molecule modifiers of miRNAs[52] have shown great promise in delivering miRNAs to specific sites or cells. Based on these advanced technologies, we anticipate that the regulation of miR-21-5p has significant therapeutic potential as a clinically viable alternative to the direct application of exogenous miRNAs and can be an effective means to improve islet transplantation outcomes in the future.

Conclusion and/or Summary

In conclusion, we demonstrated that BMSCs could deliver exosomes containing miR-21-5p into islet cells. The upregulated miR-21-5p directly binds to the 3′UTR of PDCD4, thereby reducing apoptosis in transplanted islets. This study confirms the results of previous studies, suggesting that miR-21-5p can be used as a therapeutic agent to minimize β-cell apoptosis in the early stage of islet transplantation and thus improve the transplantation outcome.

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Author Contributions

J.W., J.W.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; Y.W., R.M., S.Z.: collection and assembly of data, data analysis, and interpretation; J.Z., W.X.: conception and design, manuscript writing; X.D.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

All data generated or analyzed during this study are included in this published article (and in Supplementary Material).

Supplementary Material

Supplementary material is available at *Stem Cells* online.

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