

Mesenchymal Stem Cell-conditioned Medium Alleviates High Fat-induced Hyperglucagonemia via miR-181a-5p and its Target PTEN/AKT Signaling

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Research

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Abstract

Background: Pancreatic α -cells are critical to glucose homeostasis because they release glucagon and stimulate the liver to produce glucose. Dysregulation of α -cells gives rise to fasting and postprandial hyperglycemia in type 2 diabetes mellitus (T2DM). Mesenchymal stem cells (MSCs) or their conditioned medium can improve islet function and enhance insulin sensitivity in target tissues. However, studies showing the direct effect of MSCs on islet α -cell dysfunction are limited.

Methods: In this study, we used high-fat diet (HFD)-induced mice and α -cell line exposure to palmitate (PA) to determine the effects of bone marrow-derived MSC-conditioned medium (bmMSC-CM) involved in glucagon secretion. To investigate the potential signaling pathways, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), AKT and phosphorylated AKT (p-AKT) were assessed by Western blotting.

Results: *In vivo*, bmMSC-CM infusion protected against HFD-induced hyperglycemia and hyperglucagonemia. Consistently, bmMSC-CM decreased PA-induced glucagon secretion in α -cells and isolated islets. Additionally, bmMSC-CM reduced intracellular PTEN expression and rescued AKT signaling. Previous studies and the TargetScan database indicate that miR-181a and its target PTEN play vital roles in ameliorating α -cell dysfunction. We observed that miR-181a-5p is highly expressed in BM-MSCs but prominently lower in α TC1-6 cells. Overexpression or downregulation of miR-181a-5p respectively alleviates or aggravates glucagon secretion in α TC1-6 cells via the PTEN/AKT signaling pathway.

Conclusions: Our observations suggest that MSC-secreted miR-181a-5p mitigates glucagon secretion of α -cells by regulating PTEN/AKT signaling. These findings might provide a novel understanding of MSC-based treatment.

Introduction

Pronounced changes in lifestyle and environment have made type 2 diabetes mellitus (T2DM) a global health issue. Among adults in China, the estimated overall prevalence of diabetes is 10.9%, and that for prediabetes is 35.7% [1]. T2DM is characterized by dysfunction of pancreatic β -cells and insulin resistance. Nevertheless, α -cells cannot be underestimated because their function to release glucagon and stimulate the liver to produce glucose [2]. Dysregulation of α -cells results in fasting and postprandial hyperglycemia in T2DM [3] and is accompanied by a higher glucagon-to-insulin ratio and augmentation of the pancreatic α - to β -cell area ratio [4, 5]. In addition, there is also increased glucagon concentrations in prediabetes [6]. Fasting glucagon concentrations are associated with a longitudinal decline of β -cell function in non-diabetics [7].

Free fatty acids increase glucagon secretion in α -cell lines and isolated islets [8–12], among which α -cells exposed to fatty acids show insulin resistance of the IRS-1/PI3K/Akt pathway that likely controls glucagon secretion [12]. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a potent

negative regulator of the PI3K/AKT pathway. Its deletion in pancreatic α -cells alleviates high-fat diet (HFD)-induced hyperglucagonemia and insulin resistance [13].

Several recent clinical trials have indicated that mesenchymal stromal cell (MSC)-based therapies are effective in decreasing blood glucose and alleviating some complications such as diabetic foot injuries and diabetic retinopathy [14–18]. MSCs or their conditioned medium not only ameliorate pancreatic β -cell injury [19, 20] and islet endothelium apoptosis and functional impairment [21], but also enhance the sensitivity of insulin target tissues [22–24]. However, studies showing the direct effect of MSCs on pancreatic α -cell dysfunction are limited. An increasing number of studies indicate that the rationale for MSC therapy is based on its paracrine action rather than its differentiation mechanism [25–27]. Based on these reports, we hypothesize that MSCs could improve the function of pancreatic α -cells by secreting various factors.

Therefore, in this study, we used HFD-induced mice and α -cell line exposure to PA to investigate the effects and mechanisms of bone marrow-derived MSCs conditioned medium (bmMSC-CM) in relation to glucagon secretion.

Materials And Methods

Culture of rat bone marrow mesenchymal stem cells (BM-MSCs)

Rat primary bone marrow MSCs (BM-MSCs) were obtained by isolating the femurs of rats and flushing the marrow. Then, they were cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12) (Gibco, USA) with 20% fetal bovine serum (FBS; Gibco) supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco). The culture medium was changed every 2 days until the cells reached 80–90% confluency. After the first passage, the MSCs were cultured in DMEM/F12 with 10% FBS. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂ in air.

Identification and differentiation of BM-MSCs and preparation of MSC-conditioned medium

Flow cytometry analysis was performed to identify MSCs according to characteristics that they are positive for CD90 and CD44 (BD, USA) and negative for CD34 (BD), and CD45 (Santa Cruz Biotechnology, USA). The BM-MSCs were analyzed for their ability to differentiate into adipocytes and osteoblasts by Oil Red O (Sigma-Aldrich, USA) staining of lipid droplets and Alizarin Red S staining (Sigma-Aldrich) of calcium nodes. Once the MSCs reached 80–90% confluency, the medium was replaced with serum-free medium and harvested for 24 h. Subsequently, the supernatant was collected and concentrated 20 times using 10-kDa MW cutoff ultrafiltration membranes (Ultra-15 10K; Millipore). Finally, the bmMSC-CM was sterilized by filtration through a 0.22- μ m filter and stored at –80 °C.

Animal models and bmMSC-CM administration

Six-week-old male C57BL/6J mice were purchased from the Model Animal Research Center of Shandong University (Jinan, China) (Ethical number: DWLL-2019-016). The mice were housed with a 12-h light/dark cycle at a temperature (22–25 °C)- and humidity (55 ± 5%)-controlled environment. After one-week of adaptive feeding, the mice were randomly divided into the NCD group (which were fed a normal chow diet) and the HFD group (which were fed a 45% high-fat diet, purchased from Botai Hongda Biotechnology Co., Ltd, Beijing, China). After nine months of HFD, we separated them into two groups: HFD + PBS and HFD + bmMSC-CM. Approximately 200 µL of the bmMSC-CM concentrate, which is equivalent to a total of 100 µg of bmMSC-CM protein per mouse, were injected into the mice via their tail vein every 3 days for 10 cycles.

Animal procedures

The intraperitoneal glucose tolerance test (IPGTT) was performed after bmMSC-CM administration. Each group contained four mice selected via randomization procedure. Tail vein blood glucose levels were measured by Accu-Chek® Performa (Roche Life Science, USA) at 0, 15, 30, 60, 90, 120, and 150 min after i.p. injection of 2 g/kg body weight of glucose. The area under the curve (AUC) was calculated using the trapezoidal rule. After the mice were anaesthetized, their pancreas were fixed in 4% paraformaldehyde for immunofluorescence staining for structural assessment of islets. The sera of mice were stored at – 80 °C for glucagon enzyme-linked immunosorbent assay (ELISA; Bluegene, Shanghai, China).

Immunofluorescence staining of the pancreas

The pancreas that were fixed in 4% paraformaldehyde were embedded in paraffin, sectioned at 5-µm thickness, and mounted on glass slides. The slides were dewaxed, and then antigen retrieval was performed using antigen unmasking buffer. After blocking for 30 min at room temperature in a protein-blocking solution (10% normal goat serum), the slides were incubated with anti-mouse insulin antibody (Proteintech, China, Cat: 66198-1-Ig, 1:1,000) and anti-rabbit glucagon antibody (Proteintech, Cat:15954-1-AP, 1:200) overnight at 4 °C. The slides were then incubated with goat anti-rabbit FITC (Zhongshan, Beijing, China, Cat: ZF-0311, 1:200) and goat anti-mouse TRITC(Zhongshan, Cat: ZF-0313, 1:200) for 60 min at room temperature and then stained for DAPI for 5 min. Fluorescence was observed and captured using a fluorescence microscope (Olympus BX53, Japan). The areas of the islets, α cells, and β cells were analyzed by Image Pro Plus software. The α-or β-cell ratio in islets was measured by the glucagon or insulin-positive area divided by both-positive area (n = 3 mice and 45 islets).

α-Cell culture and processing

Palmitate (PA; Sigma-Aldrich, USA) was prepared by dissolving and heating equimolar amounts of NaOH and palmitate in distilled water to a concentration of 500 mmol/liter palmitate. This was further diluted with 5% bovine serum albumin (BSA) (fatty acid free; Sigma) to make a stock solution of 50 mmol/liter palmitate. The stock solution was filter-sterilized and stored at -20 °C, and palmitate solution was freshly prepared before each experiment.

α TC1-6 cells were obtained from the American Type Culture Collection (ATCC Number: CRL-2934™) and cultured in DMEM/high-glucose medium (Gibco, USA) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μ g/mL streptomycin at 37 °C, in a 5% CO₂ incubator. InR1G9 cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, USA) with 10% FBS and antibiotics. Both α cells were cultured to 70–80% confluency before treatment. In detail, α cells were randomly divided into the following three groups: normal control group, PA treatment group (PA, 0.5 mM), and rescue group with addition of bmMSC-CM (PA + bmMSC-CM).

Isolation and purification of islets from mice

Islets were isolated from normal C57BL/6J mice by collagenase V (1.5 mg/mL, Cat. no. C8170; Solarbio) and DNase I (62.5 U/mL, Cat. no. EN0521; Thermo Fisher) digestion followed by hand picking under a stereoscopic microscope (Olympus SZX7). Briefly, we sacrificed the mice and separated the pancreas followed by washing with ice-cold Hanks solution. Then, the pancreas were incubated in 1 mL Hanks solution that included collagenase V (1.5 mg/mL) and DNase I (62.5 U/mL) under 37°C with strong oscillation for about 12 min, until it was disintegrated into a fine sand-like suspension. Subsequently, we added 5 mL of ice-cold Hanks with 10% FBS to stop the digestion, mixed them, and then kept them on ice. After three minutes of natural settlement, we discarded the supernatant and washed the sediment with ice-cold Hanks solution thrice. To purify the isolated islets, we picked the islets into DMEM under a stereoscopic microscope. After incubation overnight, the islets were exposed to 1 mM PA and bmMSC-CM for 48 h and used to glucagon secretion assay.

Glucagon secretion

After stimulation with PA and bmMSC-CM, both of α -cells and isolated islets were incubated for 2 h in Krebs-Ringer buffer (KRB) containing 2.5 mmol/L glucose. Supernatants were collected and kept at –80 °C for glucagon ELISA.

Preparation of cell lysate and western blot analysis

After washing with ice-cold PBS, the cells were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (P0013B, Beyotime, Shanghai, China) for approximately 30 min. Then, the samples were centrifuged at 12,000 rpm at 4 °C for 15 min. Protein concentration was determined using the bicinchoninic acid (BCA) method (Beyotime, China). Subsequently, proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (EpiZyme, China) and transferred onto polyvinylidene difluoride (PVDF) membranes (IPVH00010 0.45 μ m, Millipore, USA). Then, the membranes were blocked with 5% skim milk in Tris-buffered saline solution containing Tween-20 (Sigma-Aldrich, USA) for 1 h at room temperature and incubated in specific primary antibodies at 4 °C overnight. After detection by horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature, the proteins were visualized using enhanced chemiluminescence.

The primary antibodies were as follows: PTEN (Abcam, USA, Cat. no. ab32199, 1:10000), Phosphor-AKT (CST, USA, Cat. no. 4060S, 1:2000), AKT (Abcam, Cat. no. ab179463, 1:10000), GADPH (Boster, China,

Cat. no. BA2913, 1:500).

Real-time quantitative PCR analysis

Total RNA from BM-MSCs and α TC1-6 cells was extracted using an E.Z.N.A. MicroElute Total RNA Kit (Cat. no. R6831-01; Omega BioTek, USA) following the manufacturer's instructions. Next, 1 μ g RNA was reverse-transcribed into cDNA using a Prime Script RT Reagent Kit (Cat. no. RR047A; Takara, Japan). Primers were chemically synthesized by GenePharma (Shanghai, China) Co., Ltd. The primer sequences were as follows: rno-mir-181a-5p, sense 5'-ATTCTCTCAAACATTCAACGCTG-3' and antisense 5'-TATGGTTGTTCTGCTCTCTGTGTC-3'; U6, sense 5'-CAGCACATATACTAAAATTGGAACG-3' and antisense 5'-ACGAATTTGCGTGTCATCC-3'. Real-time PCR was conducted with the SYBR Green PCR kit (Cat. no. RR420A; Takara), gene expression changes were determined with the comparative CT ($2^{-\Delta\Delta Ct}$) method, and quantification was achieved by normalization using U6 as control.

Luciferase reporter assay

Luciferase reporter assay was conducted as previously described [28]. Wild-type PTEN 3' UTR firefly luciferase reporter plasmids or PTEN 3' UTR firefly luciferase reporter plasmids with the potential miR-181a-5p binding site mutated were co-transfected with miR-181a-5p mimics or miR-NC mimics into α TC1-6 cells using Lipofectamine 2000 (Invitrogen), respectively. After 48 h of transfection, the luciferase assay was performed using a Dual-Glo Luciferase Assay Kit (Promega, Cat: E2920) according to the manufacturer's protocol.

Cell transfection

To study the effect of miR-181a-5p secreted by BM-MSCs and its target PTEN on glucagon secretion of α TC1-6 cells, we altered their expression by transfecting miR-181a-5p mimics and a negative control (NC), inhibitors and an miRNA inhibitor negative control (inhibitor NC), small interfering RNA (siRNA) for PTEN and its NC into α TC1-6 cells, respectively. All oligonucleotides were synthesized by Shanghai GenePharma Co., Ltd. Their sequences were as follows: rno-miR-181a-5p mimics, sense 5'-AACAUUCAACGCUGUCGGUGAGU-3' and antisense 5'-UCACCGACAGCGUUGAAUGUUUU-3'; NC, sense 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense 5'-ACGUGACACGUUCGGAGAATT-3'; rno-miR-181a-5p inhibitors 5'-ACUCACCGACAGCGUUGAAUGUU-3'; inhibitor NC 5'-CAGUACUUUUGUGUAGUACAA-3'; siRNA-PTEN, sense 5'-CCCACCACAGCUAGAACUUTT-3' and antisense 5'-AAGUUCUAGCUGUGGGGTT-3'; and siRNA-NC, sense 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense 5'-ACGUGACACGUUCGGAGAATT-3'.

α TC1-6 cells were transfected with miR-181a-5p oligomer and siRNA for PTEN using Lipofectamine 2000 transfection reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, the α TC1-6 cells were plated in 6-well plates until they reached 80–90% confluency. The medium was then removed and replaced by Opti-MEM I Reduced Serum Medium (Gibco) mixed with miR-181a-5p mimics (12.5 nM), inhibitor (150 nM), or siRNA for PTEN (125 nM) for 6 h. Subsequently, the culture medium was replaced by RPMI-1640 medium with 10% FBS, and the cells were treated with PA or bmMSC-CM. After 48 h, the cells and supernatant were collected for the next experiments. RT-qPCR analysis was performed to assess

transfection efficiency, whereas ELISA and western blot analysis were used for the detection of supernatant glucagon and PTEN/AKT signaling pathway, respectively.

Statistical analysis

Differences between two groups were evaluated using the *t* test; for three groups or more, a one-way ANOVA was performed. Differences between the values were considered to be significant at $P < 0.05$. All of the statistical analyses were carried out with GraphPad Prism 5, and all of the graphs were also created with this software.

Results

Identification of BM-MSCs

The bone marrow-derived MSCs (BM-MSCs) were identified by their specific surface markers, and osteogenic and adipogenic differentiation. Fluorescence-activated cell sorter (FACS) analysis showed that MSCs were positive for stem cell markers CD90 and CD44 and negative for hematopoietic markers CD34 and CD45 (Fig. 1a). After culturing in adipogenic medium for 14 days, cells differentiated toward adipocytes, which were identified by Oil Red O stain for lipid droplets (Fig. 1b). Likewise, osteogenic differentiation of MSCs was apparent after 21 days, which was determined by Alizarin Red S staining for calcium precipitation (Fig. 1c).

bmMSC-CM infusion ameliorates HFD-induced hyperglycemia and hyperglucagonemia in mice

To evaluate the effects of bmMSC-CM on pancreatic α -cells *in vivo*, we administered bmMSC-CM into HFD-fed mice. We found that bmMSC-CM infusion significantly reduced fasting glucose levels and improved the glucose tolerance during IPGTT. The AUC also supported the above findings (Fig. 2a). Circulating glucagon levels of HFD mice were higher than NCD mice, whereas bmMSC-CM infusion mitigated HFD-induced hyperglucagonemia (Fig. 2b). In addition, bmMSC-CM infusion ameliorated HFD-induced hypertrophy of islets (Figs. 2c and 2d) and decreased α and β cell area (Figs. 2e and 2f) while there was no change in the α - and β -cell ratio (Figs. 2g and 2h).

bmMSC-CM regulated palmitate-induced hypersecretion of glucagon and PTEN/AKT signaling in α -cells and isolated islets

To observe the effect of MSCs on pancreatic α -cells *in vitro*, we used bmMSC-CM to culture α TC1-6 cells, InR1G9 cells, and isolated islets of mice that were preexposed to PA. ELISA and western blotting were performed for the detection of glucagon levels in supernatants and intracellular PTEN/AKT signaling, respectively. We found that in both α -cells and isolated islets, glucagon levels in supernatants were higher in PA-preexposed cells than in the control (Figs. 3a, 3c, 3e). PA promoted the expression of PTEN and

repressed the phosphorylation of AKT (Ser473) (Figs. 3b, 3d, 3f). However, bmMSC-CM remarkably reduced PA-induced glucagon secretion (Figs. 3a, 3c, 3e). Meanwhile, in the bmMSC-CM treatment group, intracellular PTEN expression decreased, and phosphorylation of AKT(Ser473) was rescued (Figs. 3b, 3d, 3f). All of these phenomena indicated that bmMSC-CM was able to alleviate hypersecretion of glucagon and PTEN/AKT signaling transduction of α -cells.

PTEN deletion suppresses PA-induced glucagon secretion in α -cells

To determine the direct effects of PTEN on glucagon secretion in α -cells, we performed siRNA PTEN knockdown experiments using α TC1-6 cells. siRNA PTEN transfection led to efficient reduction in PTEN protein expression with increased p-Akt (Fig. 4a). Meanwhile, PA-induced glucagon secretion was attenuated after PTEN knockdown (Fig. 4b). These results suggest that PTEN/AKT signaling is involved in regulating glucagon secretion in α -cells.

Verification of miRNA secreted by BM-MSCs and its target gene

MicroRNAs (miRNAs) are important factors secreted by MSCs. To investigate the specific mechanism by which BM-MSCs regulate PTEN, we focused on miRNAs. miRNAs are small (19–24 nt) and highly conserved non-coding RNA molecules that regulate gene expression at the post-transcriptional level and play critical roles in regulating differentiation, paracrine action, and other cellular activities[29].

Studies have shown that MSCs secrete high amounts of miR-181 [30, 31]. The miR-181 family serves as a metabolic rheostat *in vivo* through the nonredundant regulation of PTEN [32]. Therefore, we first compared the differential expression of miR-181a-5p between BM-MSCs and α TC1-6 cells by RT-qPCR. We found that the mRNA level of miR-181a-5p in BM-MSCs was apparently higher than α TC1-6 cells (Fig. 5a). Furthermore, the expression of miR-181a-5p in α TC1-6 cells increased after treatment with bmMSC-CM (Fig. 5b), which indicated that miR-181a-5p secreted by BM-MSCs gain entry into α TC1-6 cells.

MiRNAs exert their functions by interacting with the 3' untranslated region (3' UTR) or protein coding sequence of target mRNAs [33]. Therefore, to further confirm that PTEN is the target gene of miR-181a-5p, we performed sequence analysis and dual-luciferase reporter assay combined with TargetScan database. Sequence analysis revealed there is a conserved miR-181a-5p binding site in the 3' UTR of PTEN mRNA (Fig. 5c), PTEN was identified as a candidate target gene of miR-181a-5p consequently, and it is associated with glucagon secretion of α -cells [13]. To explore whether miR-181a-5p directly targets PTEN, a luciferase reporter plasmid containing the predicted miRNA-binding site of PTEN(pGL3-PTEN^{WT-3'UTR}) was generated. We co-transfected with pGL3-PTEN^{WT-3'UTR} reporter plasmids and miR-181a-5p mimics or miR-NC into α TC1-6 cells, and the results showed that overexpression of miR-181a-5p inhibited the luciferase activity of PTEN 3'-UTR reporter genes. However, this inhibition was abolished when two key

nucleotides within the sequences of the putative target site in the 3'-UTR of PTEN were mutated (Fig. 5d), which suggest that PTEN may be a downstream effector of miR-181a-5p.

MSC-secreted miR-181a-5p is involved in regulating glucagon secretion and PTEN/AKT signaling in α -cells

To further corroborate the role of miR-181a-5p, α TC1-6 cells were transfected with miR-181a-5p mimics or inhibitor followed by exposure to PA or bmMSC-CM. The expression of miR-181a-5p significantly increased with the application of mimics (Fig. 6a) and reduced by inhibitor (Fig. 6d). There was a decrease in PA-induced glucagon secretion (Fig. 6b), accompanied by reduction of PTEN expression and recovery of p-AKT (Fig. 6c) after transfection with miR-181a-5p mimics. In contrast, miR-181a-5p inhibitor transfection resulted in enhanced glucagon secretion (Fig. 6e), upregulated expression of PTEN, and a decrease in p-AKT signaling (Fig. 6f). Additionally, bmMSC-CM partially rescued this process. Consequently, miR-181a-5p secreted by BM-MSCs is involved in regulating glucagon secretion and PTEN/AKT signaling of α cells.

Discussion

In this study, we investigated the possible mechanisms by which bmMSC-CM alleviates the high fat-induced α -cell dysfunction. By secreting miR-181a-5p, MSCs downregulated the expression of PTEN, a negative regulator of the PI3K/AKT pathway, and subsequently restored the phosphorylation of AKT and prevented hypersecretion of glucagon in α -cells.

Pancreatic α -cells are located in the islets of Langerhans, which constitute an important endocrine part of the pancreas. In mouse islets, approximately 10–30% of cells are α -cells, and even up to 65% of them in human islets [34, 35]. In 1948, Sutherland and Dove established that α -cells could release glucagon [36]. The main role of glucagon is to increase the release of glucose from the liver during fasting and exercise. Therefore, pancreatic α -cells play an important part in glucose homeostasis. However, in both diabetes and prediabetes, glucagon secretion is dysregulated, thereby further exaggerating hyperglycemia [3, 6, 37]. Previous *in vitro* studies showed that fatty acids stimulate glucagon secretion in isolated mouse islets and α TC1 cells, and the chain length, spatial configuration, and degree of unsaturation of fatty acids influence the glucagonotropic effect [12, 38].

Previous studies have suggested that systemic infusion of MSCs could improve islet function and ameliorate hyperglycemia [19, 20, 39]. However, studies illustrating the effect of MSCs on islet α -cells are limited. In our study, we found that bmMSC-CM infusion alleviates HFD-induced hyperglycemia and hyperglucagonemia *in vivo*. Meanwhile, hypertrophy of islets was mitigated and there was decrease in islet area, α - and β -cell area compared with HFD mice, whereas no change in α - and β -cell ratio was

detected. *In vitro*, bmMSC-CM treatment decreased PA-induced glucagon secretion in α TC1-6 cells, InR1G9 cells, and isolated islets.

Glucagon secretion by α -cells is under glucose and paracrine hormone control, in which the classical intracellular PI3K/AKT signaling pathway play a major role. Hyperglycemia increases α -cell proliferation and glucagon contents through PI3K/p-AKT changes and subsequent FoxO1 modulation [40]. Insulin from neighboring β cells directly inhibit glucagon secretion [41]. α -cells exposed to PA show impaired transduction of PI3K/AKT pathway and insulin resistance, which likely controls glucagon secretion [12]. As a potent negative regulator of PI3K/AKT pathway, upregulation of PTEN activity and transcription by PA mediate its inhibitory effects on insulin signaling [42]. PTEN deletion in β -cells improve glucose intolerance and β -cell function in HFD-fed or db/db mice [43]. PTEN deletion in α -cells protect against high-fat diet-induced hyperglucagonemia and insulin resistance [13]. *In vitro*, we found that PA promotes the expression of PTEN and inhibits phosphorylation of AKT in α -cells. Nevertheless, bmMSC-CM downregulates PA-induced PTEN expression and rescues AKT signaling.

Several studies have proposed that MSCs exert functions through modulation of autophagy [20], suppressing inflammation [23, 24], or secreting important factors such as Wnts[21]. Recently, an increasing number of studies related to MSC therapy have focus on its paracrine action[25–27]. MiRNAs are small (19–24 nt) and highly conserved non-coding RNA molecules that repress gene expression by directly binding to the 3' UTR of target messenger RNAs (mRNAs) [44]. Microarray analysis shows that miR-181 displayed abundant expression in MSC-EXO. The miR-181 family consists of six mature miRNAs, namely, miR-181a-1, miR-181a-2, miR-181b-1, miR-181b-2, miR-181c, and miR-181d, which are encoded by three independent paralog precursor transcripts on three different chromosomes [31]. Our RT-qPCR results suggest that there is prominently higher abundance of miR-181a-5p in BM-MSCs than α TC1-6 cells and that bmMSC-CM increases miR-181a-5p levels in α TC1-6 cells.

miR-181 abundance is lower in the placenta from women with pregestational overweight/obesity or gestational obesity [45]. The deregulation of miR-181 alters hepatic insulin sensitivity [46]. Henao-Mejia et al. [32] found that miR-181-deficient mice displayed robust phenotypes associated with impaired metabolic fitness, and miR-181-deficient cells displayed suboptimal glucose uptake and reduced glycolytic rates. The absence of miR-181 leads to increased PTEN activity, inhibition of the PI3K pathway activity, and therefore AKT activation. The miR-181 family acts as a metabolic rheostat *in vivo* through the nonredundant regulation of PTEN. Through overexpression and lower expression of miR-181a-5p in α TC1-6 cells, we verified its role in the regulation of PTEN/AKT signaling and secretion of glucagon. Thus, we propose that MSCs secrete miR-181a-5p, which targets PTEN/AKT signaling to alleviate the hypersecretion of glucagon in α -cells.

Conclusions

In summary, our results indicate that MSC-secreted miR-181a-5p could mitigate glucagon secretion of α -cells by regulating PTEN/AKT signaling. This is first study that shows that bmMSC-CM could alleviate

high fat-induced hyperglucagonemia in pancreatic α -cells. These findings might provide a novel understanding of MSC-based treatments.

Abbreviations

MSCs:mesenchymal stem cells; BM-MSCs:bone marrow mesenchymal stem cells; bmMSC-CM:bone marrow-derived MSC-conditioned medium; T2DM:type 2 diabetes mellitus; NCD:normal chow diet; HFD:high-fat diet; PA:palmitate; PTEN:phosphatase and tensin homolog deleted on chromosome 10; p-AKT:phosphorylated AKT; DMEM/F12:Dulbecco's modified Eagle's medium/Nutrient Mixture F-12; FBS:fetal bovine serum; IPGTT:intraperitoneal glucose tolerance test; AUC:area under the curve; ELISA:enzyme-linked immunosorbent assay; BSA:bovine serum albumin; KRB:Krebs-Ringer buffer; RIPA:radioimmunoprecipitation assay; BCA:bicinchoninic acid; NC:negative control; siRNA:small interfering RNA; miRNAs:microRNAs; 3' UTR:3' untranslated region

Declarations

Acknowledgments

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Authors' contributions

JS performed the experiments, collected data, performed data analysis, and wrote the manuscript. QH, XHG, LSW and JBW helped perform the experiments. CC, HQH, MMY and YXC helped with the sample collection. FY, JQ, ZS and XGH provided technical support and guided the data analysis. WJL and LC supervised the overall study design. All authors read and approved the final version of the manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article

Ethics approval and consent to participate

All animal-related experiments, including the isolation of rat BM-MSCs, the HFD-induced mice modeling, the bmMSC-CM infusion, and the isolation of mice islets, were conducted in accordance with the Animal Ethics Committee of Shandong University(Ethics Number:DWLL-2019-016).

Competing interests

All authors declare that they have no competing interests.

Consent for publication

All authors gave consent for publication.

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Figures

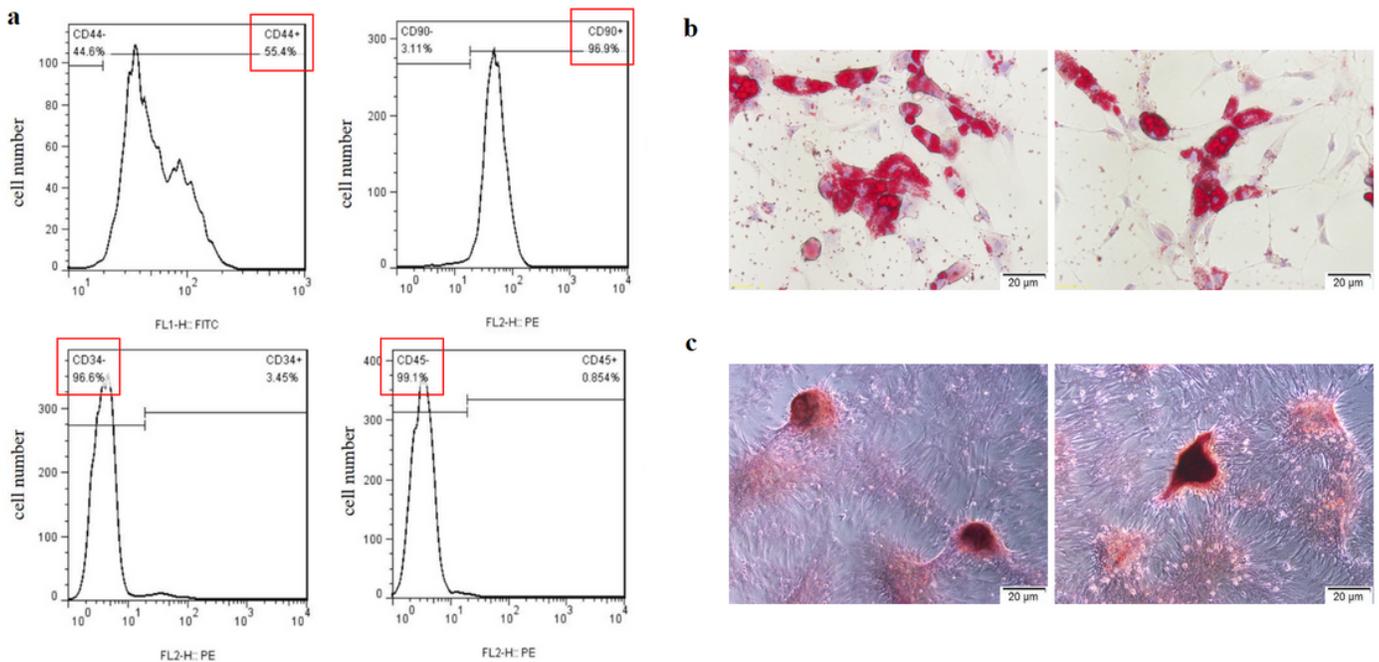


Figure 1

Identification of BM-MSCs. a. BM-MSCs were positive for stem cell markers CD90 and CD44 and negative for hematopoietic markers CD34 and CD45, which were assessed by flow cytometry analysis. b. Adipogenic differentiation was identified by intracellular lipid droplets stained in red by Oil Red O. c. Osteogenic differentiation of MSCs was identified by the formation of calcium nodes stained in red by Alizarin Red.

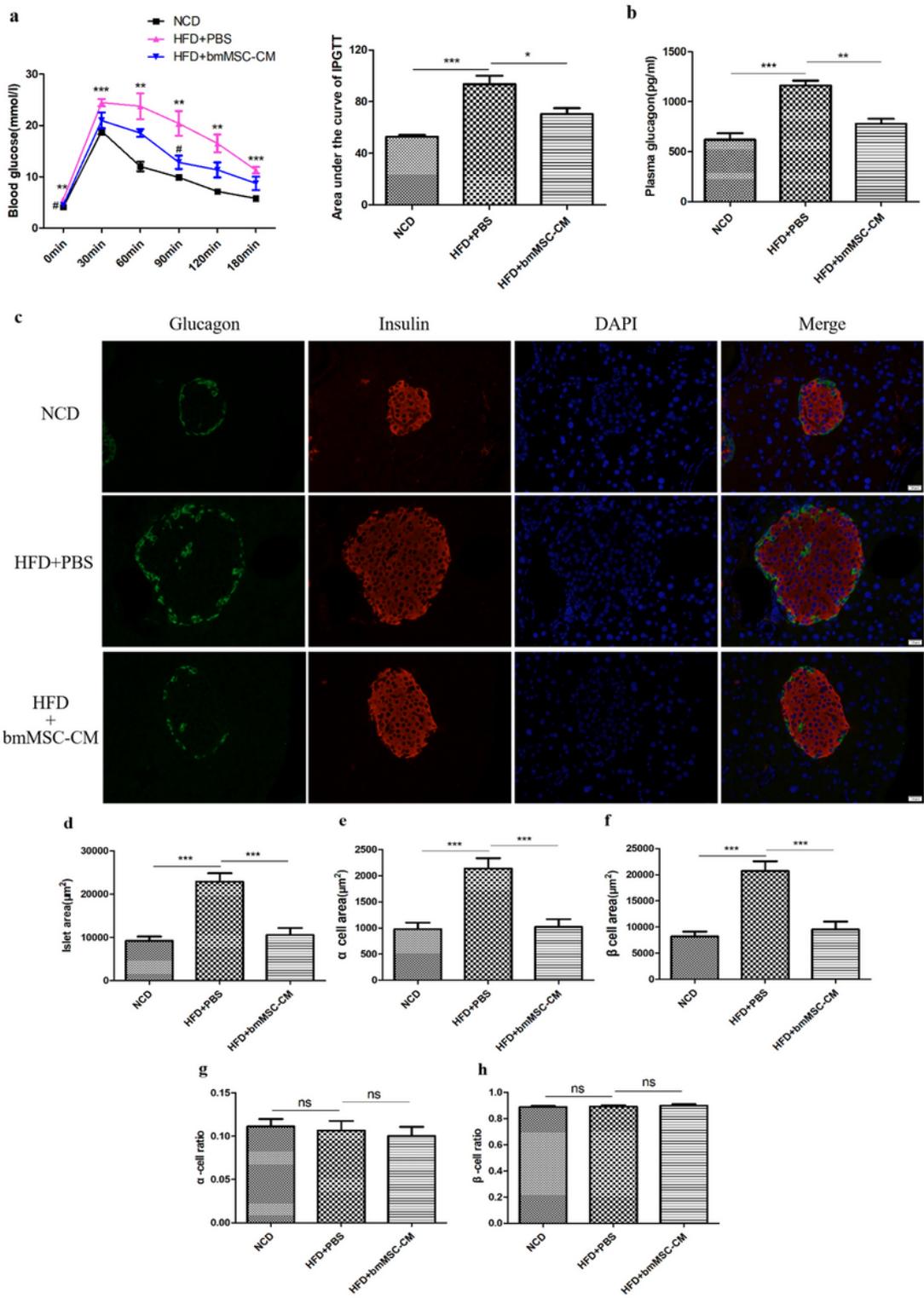


Figure 2

MSC-CM infusion ameliorated HFD-induced hyperglycemia and hyperglucagonemia in mice. a. MSC-CM infusion significantly reduces fasting glucose levels and improves glucose tolerance during IPGTT. The AUC supports the above conclusion. b. MSC-CM infusion mitigates HFD-induced hyperglucagonemia. c and d. MSC-CM infusion ameliorates HFD-induced hypertrophy of islets and decreases islet area. e and f. MSC-CM infusion decreases islet α - and β -cell area. g and h. No change in the α - and β -cell ratio is

observed (compared with the NCD group: ** $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; compared with the HFD+PBS group: # $p < 0.05$. ns: not significant).

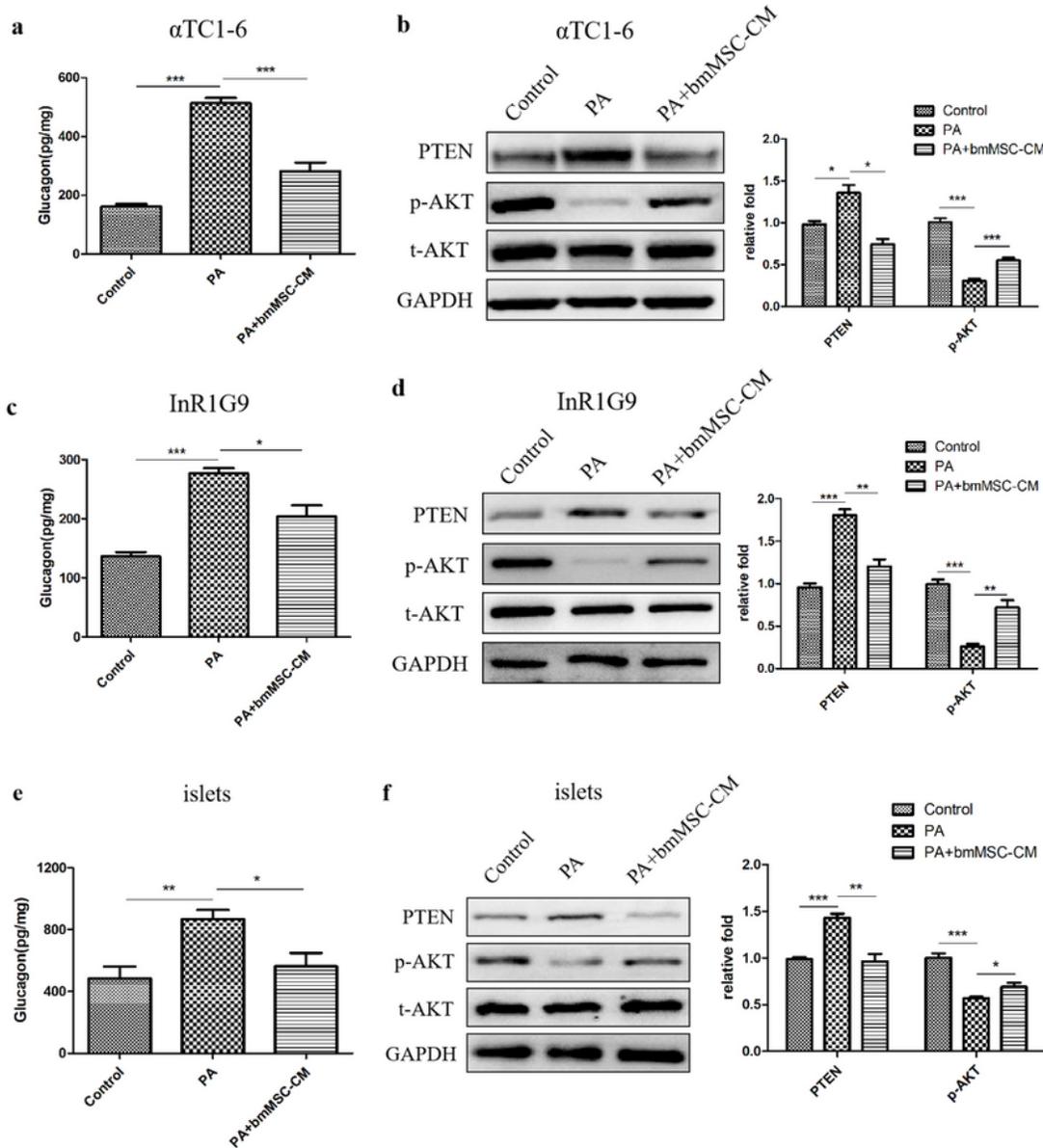


Figure 3

MSC-CM regulated palmitate-induced hypersecretion of glucagon and PTEN/AKT signaling in α -cells and islets. a, c, e. MSC-CM markedly decreases PA-induced glucagon secretion in α TC1-6 cells, InR1G9 cells and isolated islets. b, d, f. MSC-CM decreases PA-induced intracellular PTEN expression and rescues

phosphorylation of AKT (Ser473) in both α -cells and isolated islets. Quantification of bands was performed using ImageJ software (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Fig. 4 PTEN deletion suppresses PA-induced glucagon secretion of α -cells. a. siRNA PTEN transfection efficiently reduces PTEN protein expression and increases p-Akt signaling. b. PA-induced glucagon secretion is attenuated after PTEN knockdown. Quantification of bands was performed using ImageJ software (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

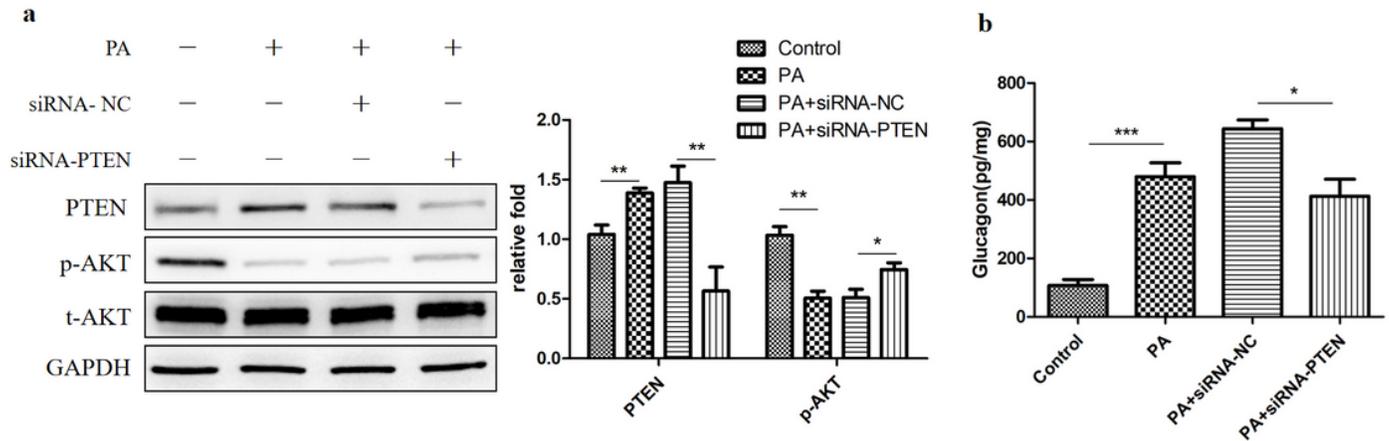


Figure 4

PTEN deletion suppresses PA-induced glucagon secretion of α -cells. a. siRNA PTEN transfection efficiently reduces PTEN protein expression and increases p-Akt signaling. b. PA-induced glucagon secretion is attenuated after PTEN knockdown. Quantification of bands was performed using ImageJ software (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

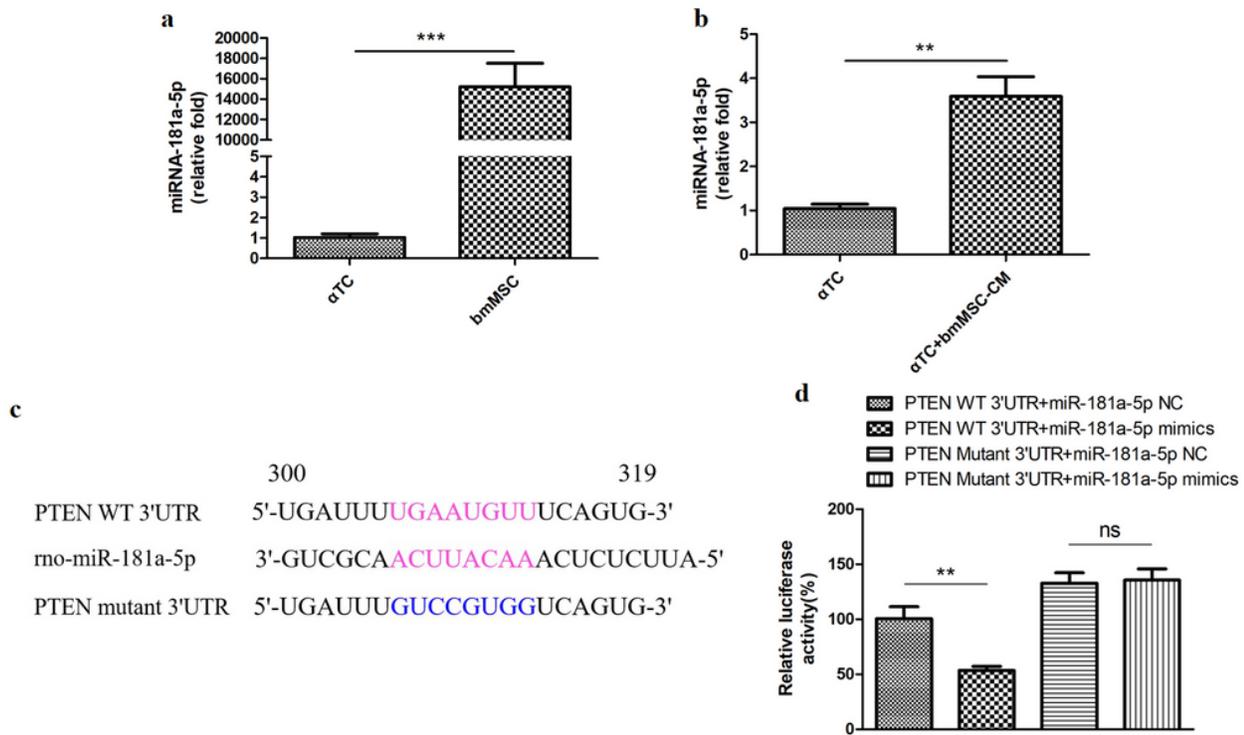


Figure 5

Verification of miR-181a-5p and its target gene. a. miR-181a-5p levels in BM-MSCs are significantly higher than αTC1-6 cells, which was confirmed by RT-qPCR. b. Upregulated expression of miR-181a-5p in αTC1-6 cells after treatment with MSC-CM. c. Schematic of the sequence that miR-181a-5p targets in the WT or mutated 3' UTR of PTEN mRNA. d. The luciferase activity of PTEN WT or mutated 3' UTR reporter plasmids in αTC1-6 cells co-transfected with miR-181a-5p mimics or miR-NC (**p<0.01; ***p<0.001; ns, not significant).

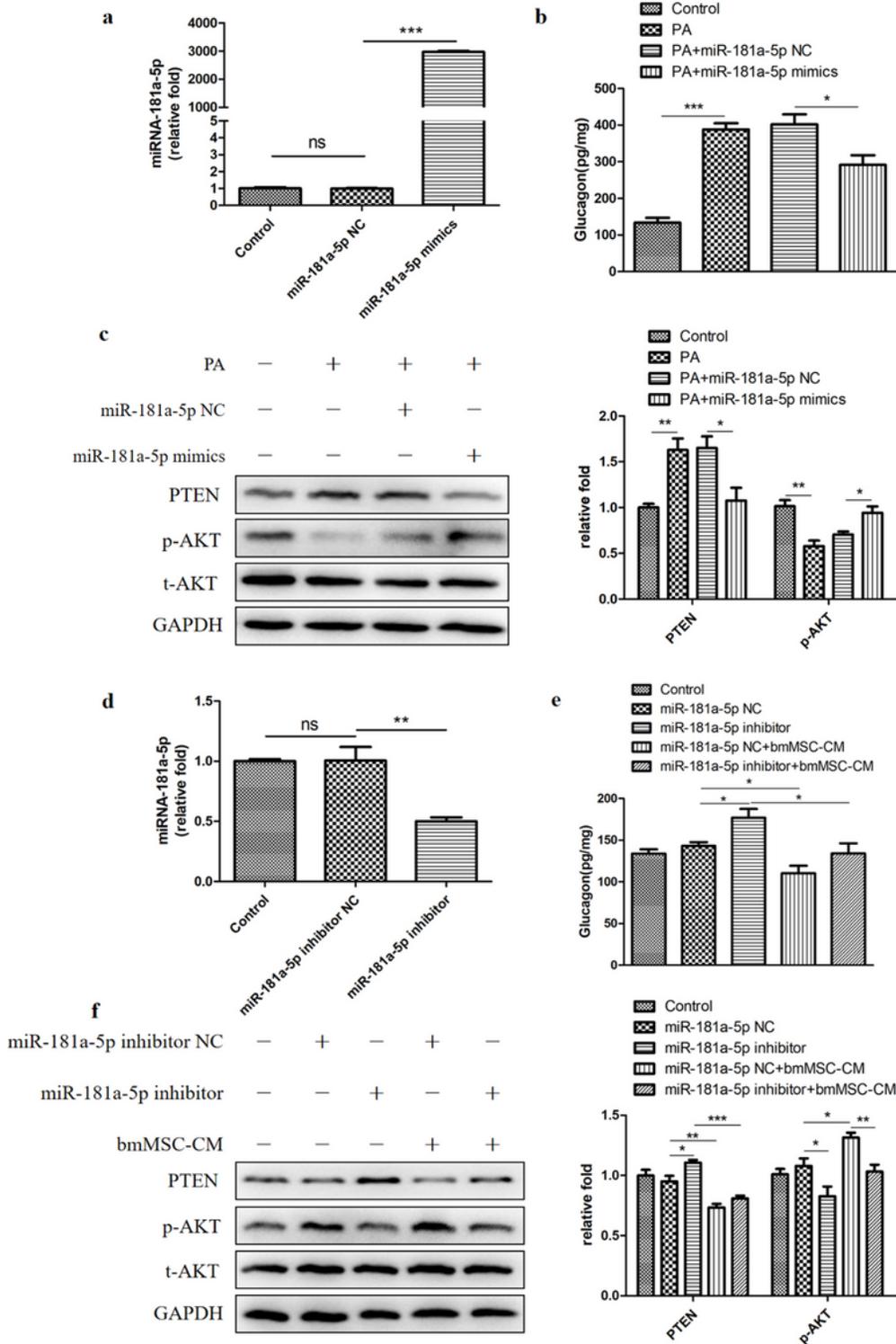


Figure 6

MSC-secreted miR-181a-5p is involved in regulating glucagon secretion and PTEN/AKT signaling in α -cells. a and d. The expression of miR-181a-5p is significantly increased by mimics (a) and reduced by inhibitor (d) as detected by qPCR analysis. b. A decrease in PA-induced glucagon secretion was observed after transfection with miR-181a-5p mimics. c. Reduction in PTEN expression and recovery of p-AKT with miR-181a-5p mimics transfection. e and f. miR-181a-5p inhibitor transfection enhances glucagon

secretion (e), upregulated the expression of PTEN and decreased p-AKT signaling (f). Additionally, MSC-CM partially rescued this process. Quantification of bands was performed using ImageJ software (*p<0.05; **p<0.01; ***p<0.001; ns, not significant).