Mesenchymal stem cell-derived exosomal miR-146a reverses diabetic β-cell dedifferentiation

Qin He 1, Jia Song 1, Chen Cui 1, Jinbang Wang 1, Huiqing Hu 1, Xinghong Guo 1, Mengmeng Yang 1, Lingshu Wang 1, Fei Yan 1, Kai Liang 1, Zhaojian Liu 2, Fuqiang Liu 1, Zheng Sun 1, Ming Dong 1,3,4, Xinguo Hou 1,3,4 and Li Chen 1,3,4,5*

Abstract

Background: Mesenchymal stem cells (MSCs) show promising therapeutic potential in treating type 2 diabetes mellitus (T2DM) in clinical studies. Accumulating evidence has suggested that the therapeutic effects of MSCs are not due to their direct differentiation into functional β-cells but are instead mediated by their paracrine functions. Among them, exosomes, nano-sized extracellular vesicles, are important substances that exert paracrine functions. However, the underlying mechanisms of exosomes in ameliorating T2DM remain largely unknown.

Methods: Bone marrow mesenchymal stem cell (bmMSC)-derived exosomes (bmMDEs) were administrated to T2DM rats and high-glucose-treated primary islets in order to detect their effects on β-cell dedifferentiation. Differential miRNAs were then screened via miRNA sequencing, and miR-146a was isolated after functional verification. TargetScan, reporter gene detection, insulin secretion assays, and qPCR validation were used to predict downstream target genes and involved signaling pathways of miR-146a.

Results: Our results showed that bmMDEs reversed diabetic β-cell dedifferentiation and improved β-cell insulin secretion both in vitro and in vivo. Results of miRNA sequencing in bmMDEs and subsequent functional screening demonstrated that miR-146a, a highly conserved miRNA, improved β-cell function. We further found that miR-146a directly targeted Numb, a membrane-bound protein involved in cell fate determination, leading to activation of β-catenin signaling in β-cells. Exosomes derived from miR-146a-knockdown bmMSCs lost the ability to improve β-cell function.

Conclusions: These findings demonstrate that bmMSC-derived exosomal miR-146a protects against diabetic β-cell dysfunction by acting on the NUMB/β-catenin signaling pathway, which may represent a novel therapeutic strategy for T2DM.

Keywords: Exosome, Mesenchymal stem cell, Type 2 diabetes mellitus, β-cell dedifferentiation, miR-146a

* Correspondence: chenli3@email.sdu.edu.cn

1Department of Endocrinology, Qilu Hospital, CheeLoo College of Medicine, Shandong University, No. 107 Wenhua Xi Road, Jinan 250012, Shandong, China

3Institute of Endocrine and Metabolic Diseases of Shandong University, Jinan 250012, China

Full list of author information is available at the end of the article

© The Author(s). 2021 Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
Background
Type 2 diabetes mellitus (T2DM) is characterized by insufficient β-cell mass and insulin resistance and has become a worldwide disease due to its increasing morbidity [1]. This T2DM-induced decline in β-cell mass is attributed to a decrease in both β-cell number and function [2]. It has been suggested that reduced β-cell mass is the result of apoptosis [3]. However, recent data challenge the β-cell apoptosis hypothesis. These data indicate that dedifferentiation represents an alternative mechanism explaining the loss of functional β-cell [4, 5]. Dedifferentiated β-cells lose their insulin secretion capacity and transform into endocrine progenitor cells with multi-directional differentiation potential, characterized by the re-expression of Ngn3, thereby causing the total amount of functional β-cells to decline [6, 7]. Interventions of β-cell dedifferentiation can reverse hyperglycemic phenotypes and slow the progression of diabetes. Therefore, safe and effective treatments that reverse β-cell dedifferentiation may represent novel and promising therapeutic strategies for T2DM.

Recently, mesenchymal stem cell (MSC)-based therapy has offered a promising strategy for treating diabetes. Some clinical studies have found that MSCs and their derivatives can significantly improve fasting blood glucose, C-peptide levels, HbA1c levels, and insulin secretion [8–10], with no significant side effects having yet been observed. Among MSCs, bone marrow MSCs (bmMSCs) have shown significant effects in regulating β-cell mass and in ameliorating chronic high-glucose-induced β-cell injury [11, 12]. Our previous research has also confirmed that bmMSCs can improve diabetic islet microcirculation via Wnt4-β-catenin signaling [13]. Although these studies have documented improved β-cell function after MSC infusion, whether decreased β-cell dedifferentiation is involved in improved β-cell mass and insulin secretion has received less attention. In the present study, we directly address this question.

Exosomes are extracellular nanoparticles with lipid-bilayer membranes [14]. Exosomes can directly deliver effectors—such as proteins, miRNAs, and microRNAs—from secretory cells to recipient cells [15, 16]. MSCs can secrete larger amounts of exosomes compared to those of terminally differentiated cells [17]. MSC-derived exosomes can reverse peripheral insulin resistance and relieve cellular destruction [18, 19]. Compared to MSCs, exosomes have lower immunogenicity and lower risk of aneuploidy and can be more easily mass-produced, all of which make them more attractive therapeutic agents. MicroRNAs are small, non-coding RNAs that exert many biological roles by negatively regulating mRNA expression at the post-transcriptional level [20]. Recent studies have demonstrated that specific miRNAs play a vital role in regulating β-cell activities and are also involved in the development of diabetic vascular complications [21]. However, it is unknown whether bmMSC-derived exosomal miRNAs affect β-cell dedifferentiation.

To elucidate the effects of bmMSCs on β cells, in the present study, we first demonstrated that bmMSC-derived exosomes (bmMDEs) reversed β-cell dedifferentiation and ameliorated β-cell function both in vitro and in vivo. miRNA sequencing was then used to further explore the role of bmMDEs. Finally, we showed that beneficial effects of bmMDEs on β-cell dedifferentiation were mainly achieved via the miR-146a-5p/Numb/β-catenin pathway. Taken together, our findings shed light on the potential of an MSC-derived exosome-based therapeutic approach for treating diabetes.

Methods
Cell culture and treatments
Rat primary bmMSCs were isolated as previously described [22]. Briefly, femurs and tibiae were obtained from Sprague-Dawley rats after euthanasia via an anesthetic overdose. The bone marrow was harvested by flushing the cavities of rats with DMEM/F12 medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS, depletion of exosomes by ultracentrifugation; Gibco). The media were changed after 24 h, and cells were at approximately 80% confluency when they were passaged. DMEM/F12 with 10% FBS was used in the subsequent cultures. The third to fourth generations of cells were used for experiments.

The INS-1 cell line was obtained from Nanjing Medical University, PR China. Cells were cultured in the RPMI-1640 medium (Gibco) supplemented with 15% FBS (depletion of exosomes by ultracentrifugation), 10 mM of Hapes (Sigma-Aldrich, St. Louis, MO), 1 mM of sodium pyruvate (Sigma-Aldrich), 2 mM of L-glutamine (Gibco), and 50 μmol/L of β-mercaptoethanol (Sigma-Aldrich) at 37°C with 5% CO₂. Cells were inoculated in a six-well plate and were cultured in a medium containing 11.1 mmol/L (control) or 35 mmol/L of glucose (high glucose, HG) for 72 h with or without INS-1 cell-derived exosomes (IDEs, 30 μg/mL, normal control exosomes) or bmMDEs (30 μg/mL). Mannitol (MA, 35 mmol/L, SM8120, Solarbio) was used as an osmotic pressure control.

Isolation and characterization of exosomes
Isolation and purification of exosomes were performed according to standard methods as described previously [23]. Briefly, the bmMSC culture medium was collected after being replaced with an exosome-free medium for 48 h. The conditioned medium was centrifuged at 300g for 10 min and was then centrifuged a second time at 2000g for 20 min to remove cells and apoptotic bodies. Next, samples were centrifuged at 10,000g for 30 min...
and were then filtered through a 0.22-μm filter to remove cellular debris. The final supernatant was centrifuged at 120,000g for 70 min at 4°C to pellet exosomes. Ultracentrifugation experiments were performed with an Optima MAX-XP (Beckman Coulter, USA). All of the exosome-containing pellets were resuspended in PBS or were lysed in RNA lysis buffer for further analysis. Exosomes were observed using a transmission electron microscope (TEM; JEM-1400, JEOL, Japan) and were quantified by a Nano-Sight NS300 (Malvern Instruments Ltd, UK).

Tracing of exosomes in INS-1 cells
Exosomes were labeled with a PKH67 Green Fluorescent Cell Linker Kit (PKH67, Sigma-Aldrich) according to the manufacturer’s protocol. First, 1 mL of Diluent C was added to the exosomes diluted in PBS. Then, 4 μL of PKH67 dye was added to 1 mL of Diluent C and was then incubated with exosomes (protected from light) for 4 min. To bind excess dye, 2 mL of 1% BSA was added to the reaction system. Re-extraction of labeled exosomes was performed by ultracentrifugation and subsequent dilution in 100 μL of PBS. The labeled exosomes were incubated with INS-1 cells. Fluorescence signals were detected by an Olympus BX53 fluorescent microscope.

Animals and diets
Four-week-old male Sprague-Dawley rats (60–70 g) were purchased from Synergy Pharmaceutical Bioengineering Co., Ltd (Nanjing, China, Ethical number: DWLL-2015-005). After 1–2 weeks of adaptive feeding, the rats were given a 45% high-fat diet (HFD) for 4 weeks, whereas the control group rats were fed with a normal chow diet. These HFD rats were then injected intraperitoneally with STZ (30 mg/kg in 0.1 M citrate-buffered saline, pH=4.5, Sigma-Aldrich) after fasting for 12 h. T2DM rats were identified as having fasting glucose ≥16.7 mmol/L. STZ-treated rats continue HFD for an additional 13 weeks. All animal experimental protocols were approved by the Animal Ethics Committee of Shandong University.

Analysis of metabolic parameters
Rats were fasted for 3 h before measuring blood glucose levels. Body weight (BW) and food intake were monitored weekly. Intraperitoneal glucose tolerance tests (IPGTTs) and intraperitoneal insulin tolerant tests (IPITTs) were performed after the last intervention. IPGTTs were performed following overnight fasting. Blood glucose levels were detected at 0, 30, 60, 120, and 180 min after intraperitoneal injections of glucose (1.5 g/kg, Sigma-Aldrich). For IPITTs, rats were provided free access to food and water. Blood glucose levels were measured at 0, 30, 60, 120, and 180 min after intraperitoneal injections of insulin (2 IU/kg, Wanbang Pharmaceutical, Jiangsu, China).

Isolation and intervention of islets from rats
Pancreatic tissues from rats were isolated after anesthesia. A syringe was pumped with 1 mg/mL of Collagenase V (C9263; Sigma-Aldrich) and was then replaced with a fine syringe needle inserted into the pancreas. The outflow of the enzymatic solution was re-injected 2–3 times. The harvested pancreas was then placed into a digestive bottle, and it was then digested at 37°C for 15 min. Subsequently, the sample was removed from the water bath and was lightly shaken 12 times. Ice-cold Hanks solution (with calcium and magnesium) was added to the digestive bottle and was shaken to stop collagenase digestion, after which the sample was placed on ice three times. Finally, the clean islets were handpicked under a stereoscopic microscope. After preincubation overnight, the islets from Sprague-Dawley rats were exposed to 5.0 mmol/L (control) or 35 mmol/L of glucose (high glucose, HG) with or without IDEs (30 μg/mL) or bmMDEs (30 μg/mL) for 72 h. The islets from control, T2DM, and T2DM + bmMDE rats were pre-cultured overnight, after which they directly proceeded to the next experiment. MA (35 mmol/L) was used as an osmotic pressure control.

Glucose-stimulated insulin secretion (GSIS)
After the corresponding intervention was completed, glucose-starved INS-1 cells and islets (50–60 islets in each group) were assessed in Krebs–Ringer bicarbonate solution (KRBS) containing 2.5 mmol/L of glucose at 37°C for 1 h. The supernatant was collected before being incubated in KRBS containing 25 mmol/L of glucose for 30 min.
another 1 h at 37°C. The supernatant was collected and insulin levels were detected using a commercial kit (Blue Gene, Shanghai, China) to evaluate stimulated insulin release. The final insulin content was normalized to the protein concentrations of cells and islets.

**Immunofluorescence staining**

INS-1 cells and paraffin sections of pancreatic tissue were incubated with the following antibodies: insulin (ab7842; Abcam), glucagon (ab10988; Abcam), PDX1 (5679; Cell Signaling Technology), FOXO1 (2880; Cell Signaling Technology), neurogenin3 (NGN3, sc-3744042; Santa Cruz, USA), OCT4 (GTX101497; Gene Tex, CA, USA), and NUMB. After incubated with the fluorescence-conjugated secondary antibodies, the cell nuclei were stained with DAPI. Digital images were captured using an Olympus BX53 fluorescence microscope. Immunofluorescence staining statistics are measured from 3 to 5 rats per group, or 4–5 pancreas sections per rat for 15–20 islets. Quantitative analysis was performed using the CytosNuclear count function of the Image Pro-Plus software. Mean β-cell percentage per islet = average (β-cell number/total islet cell number) × 100%. Densitometry of staining was performed using ImageJ. Images were separated by color and inverted to black and white, and the integrated density was calculated for each stain using the tracing function.

**Real-time quantitative PCR (RT-qPCR)**

Total RNAs were extracted from INS-1 cells and islets using MicroElute Total RNA Kit (Omega Bio-Tek, Denville, GA, USA). Total miRNAs were extracted by a miRcute miRNA isolation kit (TIANGEN Biotech, Beijing, China) according to the manufacturer’s instructions. The sequences of above primers are listed in Supplementary Table 1. Real-time PCR was conducted with the SYBR Green PCR kit (catalog no. RR820B; Takara), and quantification was achieved by normalization using β-actin or U6 as the control.

**Small RNA library construction and sequencing**

Total RNA was extracted using Trizol reagent (Invitrogen, CA, USA) following the manufacturer’s procedure. The total RNA quantity and purity were analyzed with a Bioanalyzer 2100 and an RNA 6000 Nano LabChip Kit (Agilent, CA, USA) with RIN number >7.0. Approximately 1 μg of total RNA was used to prepare a small RNA library according to the protocol of TruSeqTM Small RNA Sample Prep Kits (Illumina, San Diego, USA). Additionally, single-end sequencing (36 bp) was performed on an Illumina Hiseq2500 Lianchuan Biotechnology (LC-BIO, Hangzhou, China) following the vendor’s recommended protocol.

**Luciferase reporter assay**

To identify the binding site between miR-146a and Numb, INS-1 cells were transfected with a luciferase construct containing Numb with the wild-type or a mutated version of the binding site and then co-transfected with miR-NC or miR-146a mimics (GenePharma, Shanghai, China). Luciferase activities were detected after 48 h of transfection by using a Dual-Luciferase kit (Promega, Madison, USA) according to the manufacturer’s instructions.

**RNA interference and plasmids**

siRNAs (siNC, siNUMB), plasmid vectors (vector containing Numb and empty vector), mimics/inhibitors of indicated miRNAs, and lentiviral vectors (vectors containing miR-146a sponge inhibitor and control) were prepared by GenePharma (Shanghai, China). The target sequence of Rattus-Numb (NM_133287.1) was 1799 bp, the vector was pcDNA3.1(+), and the restriction site was NheI-NotI. The sequences of siRNAs and miRNA mimics/inhibitors referred above are listed in the Supporting Information, namely in Tables S1 and S2.

**Statistical analysis**

Three independent experiments were performed. The results are expressed as the mean ± the standard deviation (SD). Data were compared using paired Student’s t tests or one-way analyses of variance (ANOVAs) followed by Bonferroni tests in GraphPad Prism 8 software (San Diego, CA, USA). A P-value < 0.05 was considered statistically significant ( *P*<0.05; **P**<0.01; ***P***<0.001).

**Results**

bmMDEs improve pancreatic islet functions in T2DM rats

bmMSCs were identified by the expression of surface markers (negative for CD45 and CD34, and positive for CD105, CD73, and CD90) (Fig. S1c), as well as by differentiation capacities toward adipocytes confirmed by Oil-Red-O staining (Fig. S1a) and toward osteoblasts confirmed by Alizarin Red staining (Fig. S1b). bmMDEs were isolated using standard differential centrifugation from cell culture supernatants. The cup-shaped membranous structure, size, and number of exosomes were identified by transmission electron microscopy (TEM) (Fig. 1a) and with a mode diameter around 100 nm via a nanoparticle tracking system (Fig. 1a, b). In addition, isolated exosomes were characterized using Western blotting to determine the presence of the established exosomal protein markers, CD9 and TSG101. Calnexin, an endoplasmic reticulum marker, was present in cells but not in exosomes (Fig. 1c). To address the biodistribution of exosomes in rats, PBS and Cy7-labeled bmMDEs were injected into each rat via the tail vein.
Fig. 1 (See legend on next page.)
Rats were imaged at 24 h after injections using an in vivo imaging system (IVIS) (Fig. 1d). Organs were then harvested and imaged ex vivo to clarify fluorescent signals and minimize interference (Fig. 1e).

To evaluate the therapeutic effects of bmMDEs on T2DM, we used a rat model of T2DM based on a high-fat diet combined with STZ injections. Blood glucose was significantly reduced after bmMSC intervention (5×10⁶ cells/rat, once every 2 weeks) compared to that in the control group (Fig. 1j). Additionally, bmMDEs (10 mg/kg, once every 3 days) also greatly ameliorated body weight of T2DM rats compared with those in islets from diabetic rats. In addition, the expression levels of NGN3 and OCT4 in diabetic islets were markedly decreased after bmMDE treatment. Glucose-stimulated insulin secretion (GSIS) results showed that bmMDEs improved glucose-stimulated insulin release in diabetic islets (Fig. 2c). The mRNA levels of Pdx1, Foxo1, Ngn3, and Oct4 were also consistent with the results of immunofluorescent staining (Fig. 2d).

High-glucose-induced β-cell loss commonly leads to islet dysfunction. Therefore, we investigated the effects of bmMDEs on β-cell dysfunction in primary islets and in the INS-1 cell line. First, we used mannitol (MA) as an osmotic pressure control to eliminate the effects of osmotic pressure in HG environments. The results showed that MA had no significant effect on insulin secretion or β-cell dedifferentiation in islets and INS-1 cells, while exposure to HG environment led to a significant decrease in glucose-stimulated insulin release (Fig. S2a–d). The expression levels of Pdx1 and Foxo1 were reduced after HG stimulation in islets and INS-1 cells, and the levels of Ngn3 and Oct4 were obviously elevated in islets but not significantly different in INS-1 cells from those in the normal control group (Fig. S2–c, d), which indicated that HG-induced INS-1 dedifferentiation was relatively insufficient. Next, bmMDEs were labeled with PKH67 green and were co-cultured with INS-1 cells, after which cytoskeletons were visualized using incubation in rhodamine phalloidin for 24 h to detect exosomal uptake in INS-1 cells (Fig. S2–e). GSIS results showed that bmMDEs improved the reduction of glucose-stimulated insulin release in islets (Fig. 2e) and INS-1 cells (Fig. S2–f). Real-time quantitative PCR (RT-qPCR) analysis showed that bmMDEs could upregulate the expression of Pdx1, Foxo1 in HG-induced islets and INS-1 cells, and downregulate the expression of Ngn3 and Oct4 in islets (Fig. 2f, Fig. S2–g).

In all of the above results, normal control exosomes (IDEs) had no effects in HG-treated islets or INS-1 cells.
These data suggest that treatment with bmMDEs reversed β-cell dedifferentiation both in vivo and in vitro.

**MiR-146a in bmMDEs reverses β-cell dedifferentiation**

We next explored how bmMDEs reversed dedifferentiation of β-cells. Exosomes can regulate physiological activities through exosomal miRNAs [25, 26]. We therefore conducted high-throughput sequencing of miRNA profiles in IDEs, HG-treated INS-1-cell-derived exosomes (HIDEs), and bmMDEs. A heat map was created to identify the differentially abundant miRNAs (Fig. 3a), and 10 of the most upregulated miRNAs in bmMDEs were subjected to validation by RT-qPCR. The expression levels of all 10 miRNAs in bmMDEs were

![Image](https://example.com/image.png)
higher than those in IDEs and HIDEs (Fig. 3b). We then performed a screen for the functional miRNA using the GSIS experimental setup in INS-1 cells. After pretreatment with top 10 miRNA mimics, the cells were then incubated with HG medium for 72 h. As shown in Fig. 3c, miR-146a-5p (miR-146a) mimics significantly increased insulin secretion that was otherwise inhibited by high-glucose environments compared to those of the other nine miRNA mimics. The overexpression efficiencies of miRNAs are shown in the Supporting information, namely in Fig. S3a. In addition, miR-146a mimics also ameliorated deleterious effects of HG in islets (Fig. S3b, c). RT-qPCR analysis further confirmed that treatment with bmMDEs elevated the expression level of miR-146a in both INS-1 cells and primary cultured islets (Fig. 3d, e). Furthermore, expression levels of Pdx1 and Foxo1 increased, while those of Ngn3 and Oct4 decreased in the HG + miR-NC-mimic group, compared with those in the HG + miR-NC-mimic group in primary cultured islets (Fig. 3f–i). To further investigate the role of miR-
Numb showed that interfering RNAs (siRNAs) in islets. GSIS experiments overexpressed full-length Numb. Furthermore, to determine the function of NUMB on islets, Numb mimics significantly reduced luciferase activity in INS-1 cells generated. We then found that miR-146a, a human homolog membrane-bound protein [27], was noteworthy. It is known that NUMB is upregulated in diabetic patients and positively regulates the expression of NGN3, a marker of islet progenitor cells [28–30]. We found that the expression level of Numb was significantly upregulated in islets within T2DM rats compared with that in the control group, and that bmMDE treatment decreased NUMB expression (Fig. 4a; Fig. S4a). We then confirmed that Numb expression was enhanced after HG treatment and was downregulated by an miR-146a mimic at the mRNA level in islets (Fig. 4b), as well as at the protein level in INS-1 cells (Fig. 4c; Fig. S4b). Subsequently, we found that bmMDEs reduced the expression of Numb post-HG treatment, whereas an miR-146a inhibitor attenuated this effect of bmMDEs in terms of mRNA in islets (Fig. 4d) and at the protein level in INS-1 cells (Fig. 4e; Fig. S4c). Through bioinformatic analysis, we found that the 3′ UTR of the Numb gene contained putative binding sites for miR-146a (Fig. 4f). To further verify the association between Numb and miR-146a, luciferase reporter plasmids containing the wild-type 3′ UTR (Numb 3′ UTR WT) and mutated 3′ UTR (Numb 3′ UTR MUT) of Numb were generated. We then found that miR-146a mimics significantly reduced luciferase activity in INS-1 cells containing Numb 3′ UTR WT but had no significant effect on Numb 3′ UTR MUT cells (Fig. 4g). Furthermore, to determine the function of NUMB on islets, we overexpressed full-length Numb (Fig. S4d, e) and knocked down Numb expression (Fig. S4f, g) with small interfering RNAs (siRNAs) in islets. GSIS experiments showed that Numb overexpression reduced GSIS, and inhibition of Numb attenuated the inhibition of GSIS by the HG environment (Fig. 4h, m). Finally, we examined the effect of NUMB on the dedifferentiation of islet β-cells in vitro. RT-qPCR results showed that the expression levels of Pdx1 and Foxo1 were significantly decreased after Numb overexpression and that the expression levels of Ngn3 and Oct4 were significantly increased (Fig. 4i–l). The effect achieved after suppressing Numb was similar to that of miR-146a mimics shown in Fig. 3f–i (Fig. 4n–q). These data demonstrate that Numb is the direct downstream target of miR-146a-5p in the regulation of β-cell function and dedifferentiation.

**MiR-146a/Numb improves β-cell function through β-catenin nuclear translocation**

β-catenin is a known signaling molecule that is downstream of Numb [31]. β-catenin has been shown to modulate pancreatic ontogenesis and islet function [32, 33]. Therefore, we explored whether β-catenin mediates the ameliorative effects of bmMDEs in our T2DM model in vitro. First, we examined the protein levels of β-catenin under different intervention conditions. We found an increase in nuclear protein levels of β-catenin in islets treated with bmMDEs, whereas the protein level of β-catenin in the cytoplasm was not changed (Fig. 5a, Fig. S5a). Similar results were examined in the miR-146a-mimic group (Fig. 5b; Fig. S5b). The effect of bmMDEs increasing the nuclear protein level of β-catenin protein was attenuated when the expression of miR-146a was inhibited (Fig. 5c; Fig. S5c). To confirm activation of the β-catenin signaling pathway, XAV-939, a β-catenin inhibitor, was used to promote β-catenin degradation. GSIS was impaired after XAV-939 intervention, and miR-146a mimics effectively attenuate this damage (Fig. 5d). We then found that miR-146a mimics elevated the expression levels of Pdx1 and Foxo1 and decreased expression levels of Ngn3 and Oct4 in HG-treated islets. However, the effect of miR-146a mimics was partially restored when combined with XAV-939 (Fig. 5e–h). Then, we verified the relationship between Numb and β-catenin in primary islets and INS-1 cells, respectively. The nuclear protein levels of β-catenin decreased after Numb overexpression, while these levels were elevated after knock down of Numb (Fig. 5i, j and Fig. S5d–g). These results suggest that Numb/β-catenin may be downstream effectors of miR-146a in the regulation of islets and β-cell function and dedifferentiation.

**MiR-146a knockdown abolishes bmMDE-mediated reversal of β-cell dedifferentiation in vivo**

To test whether miR-146a is required for the efficacies of bmMDEs in vivo, we knocked down miR-146a expression via lentiviruses in bmMSCs (Fig. S6a). As expected, the level of miR-146a in exosomes derived from miR-146a-knockdown bmMSCs (miR-146a KD bmMDEs) was decreased significantly compared with that from exosomes derived from miR-NC-knockdown bmMSCs (miR-NC KD bmMDEs) (Fig. S6b). Exosomes were then injected into T2DM rats to detect their effects on β-cell function in vivo. Levels of blood glucose rose to ~18 mmol/L at week 5 in all rats after STZ injection. However, blood glucose of rats decreased dramatically after bmMDE intervention in the T2DM + bmMDEs-miR-NC KD group (~10 mmol/L at week 10), as compared
Fig. 4 Exosomal miR-146a directly targets Numb in β-cells. a Immunofluorescent staining for NUMB expression in pancreatic sections (scale bar 20 μm). RT-qPCR analyses of the mRNA levels of Numb in islets transfected with miR-146a mimics (b) or treated with an miR-146a inhibitor combined with bmMDEs (d). Western blotting for NUMB in INS-1 cells transfected with miR-146a mimics (c) or treated with an miR-146a inhibitor combined with bmMDEs (e). β-actin was used as the loading control. f Schematic of the sequence that miR-146a targets in the WT or mutated 3′ UTR of Numb mRNA. g Luciferase activity of Numb WT or mutated 3′ UTR reporter plasmids in INS-1 cells transfected with miR-146a mimics or miR-NC mimics as indicated. A GSIS assay was performed to detect insulin stimulation in islets transfected with Numb overexpression (h) or siRNA (m). RT-qPCR analyses for Pdx1, Foxa1, Ngn3, and Oct4 in islets transfected with Numb overexpression (i-l) or siRNA (n-q). Data are presented as the mean ± SEM (*P < 0.05; **P < 0.01; ***P < 0.001)
with that in the T2DM + PBS group (~18 mmol/L at week 10) and T2DM + bmMDEs\textsuperscript{miR-146a KD} group (~16.5 mmol/L at week 10, Fig. 6a). The body weight of T2DM rats was significantly increased after bmMDEs\textsuperscript{miR-NC KD} treatment, but there was no statistical difference compared with that in the bmMDEs\textsuperscript{miR-146a KD} treated group (Fig. S6c). T2DM rats treated with bmMDEs\textsuperscript{miR-NC KD} showed significant improvement in glucose tolerance and insulin sensitivity, and, as expected, the effect of bmMDEs was abolished via miR-146a knockdown (Fig. 6b–e). Moreover, serum insulin levels in the T2DM + bmMDEs\textsuperscript{miR-NC KD} group were significantly higher than those in the T2DM + PBS group and T2DM + bmMDEs\textsuperscript{miR-146a KD} group (Fig. 6f). Next, immunofluorescent staining was performed in pancreatic tissue sections from bmMDE-treated rats to examine dedifferentiation of islets in vivo. The expression levels of PDX1 and FOXO1 were increased in islets, Fig. 5 MiR-146a/Numb reverses β-cell dedifferentiation and improves β-cell function through β-catenin nuclear translocation. a Nuclear translocation of β-catenin detected after bmMDE treatment in primary islets, as indicated by Western blotting analysis. b, c Western blotting for nuclear translocation of β-catenin in primary islets transfected with miR-146a mimics or treated with an miR-146a inhibitor combined with bmMDEs. Glucose-stimulated insulin secretion (d) in islets transfected with miR-146a mimics combined use XAV-939. e–h RT-qPCR analyses for Pdx1, Foxa1, Ngn3, and Oct4 in islets transfected with miR-146a mimics combined use XAV-939. Confirmation of the changes of β-catenin nuclear translocation of Numb overexpression (i) and siRNA (j). β-actin and GAPDH were used as loading controls. Data are presented as the mean ± SEM (\(\ast P < 0.05; \ast\ast P < 0.01; \ast\ast\ast P < 0.001\)).
while those of NGN3 and OCT4 were decreased in T2DM rats after bmMDE miR-NC KD treatments compared with those in the T2DM + PBS group and T2DM + bmMDEs miR-146a KD group (Fig. 6g; Fig. S6d). RT-qPCR results of corresponding primary cultured islets demonstrated that bmMDEs upregulated the expression levels of Pdx1 and Foxo1 and decreased expression levels of Ngn3 and Oct4, while the effects of bmMDEs were abolished by miR-146a knockdown in T2DM rats (Fig. 6h–k). These data indicate that exosomes without miR-146a attenuated their ability to improve β-cell function and reverse dedifferentiation.
Discussion

The declining function of pancreatic β-cells is central to the progression of T2DM, and it has been confirmed that β-cell dedifferentiation is an important mechanism leading to functional β-cell reduction [5, 34, 35]. In recent years, MSC-based therapies have been demonstrated as potential approaches for treating diabetes and other diseases [36, 37]. However, some disadvantages of MSCs—such as their tumorigenic potential, issues in inducing thrombosis, and low survival times in vivo—have limited their clinical applications. Increasing evidence has suggested that exosomes, an important manifestation of paracrine actions, are the underlying substances that mediate the therapeutic effects of MSCs [38–40]. Our present results also confirmed that bmMDEs had similar therapeutic effects to those of bmMSCs on islet functions.

Hyperglycemia-induced dedifferentiation is an important mechanism that leads to β-cell failure [35, 41–43], and we found that β-cells in diabetic rats and primary islets with high glucose interventions lost their identities and turned into pancreatic endocrine progenitors that featured by high levels of Ngn3 and Oct4, as well as significantly decreased expression of Pdx1 and Foxo1. These results are consistent with previous reports [6, 44]. However, in the bmMDE treatment group in our present study, β-cell markers were restored and dedifferentiation markers were suppressed, indicating that bmMDEs may alleviate the dedifferentiation of β-cells. These findings suggest novel treatment effects of bmMDEs in mitigating hyperglycemia-induced β-cell dysfunction.

Exosomes regulate many physiological activities through exosomal miRNAs [37, 45]. In our present study, miR-146a emerged from our non-biased sequencing and subsequent functional screen. Interestingly, miR-146a has previously been characterized in the context of T2DM [46, 47]. For example, miR-146a is significantly lower in diabetic patients than in healthy controls and is significantly overexpressed in T2DM patients treated with metformin [48]. Liu et al. found that miR-146a mimics improve diabetic retinopathy by regulating IRAK1/TRAf6 and their downstream pro-inflammatory gene expressions [47]. Beatrix et al. discovered that miR-146 is a potential therapeutic target for the treatment of diabetic retinopathy through its inhibition of NF-κB activation [49]. Roggli et al. indicated that miR-146a was up-regulated in human pancreatic islets after exposure to pro-inflammatory cytokines, and inhibition of miR-146a leads to improved β-cell function [50]. In addition, Anna et al. showed that compared with human pancreatic islets (24 h), INS-1 cells have a rapid (<6 h) expression of inflammatory genes induced by cytokine signaling, and the expression of miR-146a is downregulated upon
exposure in INS1 cells after 24 h. Transfection of INS1 cells with miR-146a-5p also downregulates islet inflammation and β-cell death in part by impaired NF-κB and MAPK signaling [51]. In our study, high glucose intervention for 72 h is a chronic stimulus, and the decrease in miR-146a-5p expression may be a combination of alterations secondary to changes in the transcription of early immediate response genes and compensatory/restorative responses. However, further verification in human pancreatic islets or β-cell derived from hiPSC/hESC is still needed to make the conclusion more convincing.

Next, we discussed the specific role and mechanism of miR-146a in regulating β-cell dedifferentiation. After consulting the TargetScan database and previous studies, we found that the miR-146a downstream target gene, Numb, is upregulated in diabetic patients and positively regulates the expression of the islet progenitor cell marker, NGN3. Here, we then indicated that miR-146a negatively regulated β-cell dedifferentiation by directly binding to 3' UTR of Numb mRNA and inhibited its expression in β-cells. Even though the association between miR-146a and Numb has been reported previously [52–54], our study demonstrated, for the first time, that bmMDE-derived exosomal miR-146a rescued diabetic β-cell dysfunction via Numb inhibition.

β-catenin has been reported to be a downstream target gene that is negatively regulated by Numb and is linked to the regulation of islet function [54, 55]. To further understand the mechanism underlying Numb-mediated responses, we investigated β-catenin signaling in the context of diabetic β-cell dysfunction. We demonstrated that bmMDE treatments or miR-146a mimics enhanced β-catenin nuclear translocation. Moreover, miR-146a mimics rescued the inhibition of β-catenin, islet dysfunction, and dedifferentiation caused by XAV-939. We also verified the targeting relationship between Numb and β-catenin. These results highlight that the miR-146a/Numb/β-catenin signaling pathway is a crucial mediator of bmMDE-induced amelioration of hyperglycemia-induced β-cell dysfunction.

Conclusion
In summary, our present findings elucidate a novel mechanism by which bmMDEs regulate islet function and β-cell dedifferentiation. Results from T2DM rats and primary islets indicated that the beneficial effects of bmMDEs were mainly achieved via the miR-146a-5p/Numb/β-catenin pathway (Fig. 7). Our findings shed light on the potential of MSC-derived exosome-based therapeutic approaches for treating diabetes.

Abbreviations
MSCs: Mesenchymal stem cells; bmMSC: Bone marrow mesenchymal stem cell; bmMDEs: Bone marrow mesenchymal stem cell-derived exosomes; IDEs: INS-1-derived exosomes; T2DM: Type 2 diabetes mellitus; IPGT: Intrahepatic pancreatic islets or β-cell dedifferentiation. Results from T2DM rats and primary islets indicated that the beneficial effects of bmMDEs were mainly achieved via the miR-146a-5p/Numb/β-catenin pathway (Fig. 7). Our findings shed light on the potential of MSC-derived exosome-based therapeutic approaches for treating diabetes.

Acknowledgements
We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

Authors’ contributions
QH performed the research, data analysis, and manuscript writing. JS, CC, JBW, HQH, XHG, and MMY participated in the research and data collection. LSW, FY, and KI helped with the sample collection. ZJL and FQL contributed to the data analysis. ZS provided technical support and edited the paper. LSW, FY, and KL helped with the sample collection. ZJL and FQL contributed to the data analysis. ZS provided technical support and edited the paper. XGH and MD contributed to the design of the study. LC supervised the overall study design. All authors read and approved the final manuscript.

Funding
This work was supported by the National Natural Science Foundation of China (grant numbers 82078880, 81670706, 81873632, 81770818, 81800736, 81800727, 81900756), the National Key R&D Program of China (grant numbers 2016YFC0901204, 2018YFC1311801), the Taishan Scholars Foundation of Shandong Province (grant number ts201712089), and the Natural Science Foundation of Shandong Province (grant number ZR2019PH018, ZR2019PH078).

Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
All animal experiments were conducted in accordance with the Animal Ethics Committee of Shandong University (ethical number DWLL-2015-005).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1Department of Endocrinology, Qilu Hospital, Cheelu College of Medicine, Shandong University, No. 107 Wenhua Xi Road, Jinan 250012, Shandong, China. 2Department of Cell Biology, Cheelu College of Medicine, Shandong University, Jinan 250012, China. 3Institute of Endocrine and Metabolic Diseases of Shandong University, Jinan 250012, China. 4Key Laboratory of Endocrine and Metabolic Diseases, Shandong Province Medicine & Health, Jinan 250012, China. 5Jinan Clinical Research Center for Endocrine and Metabolic Diseases, Jinan 250012, China.
References

1. Classification and Diagnosis of Diabetes. Standards of Medical Care in Diabetes-2021. Diabetes care. 2021;44(Supplement 1):s1–s33. https://doi.org/10.2337/dc21-5002.

2. Prentki M, Matschinsky FM, Madiraju SR. Metabolic signaling in fuel-induced insulin secretion. Cell metabolism. 2013;18(2):162–85. https://doi.org/10.1016/j.cmet.2013.05.018.

3. Tomita T. Apoptosis in pancreatic β-cell islets in type 2 diabetes. Bosnian journal of basic medical sciences. 2016;16(3):162–79. https://doi.org/10.173/05/bjbsm.2016.919.

4. Diedsheim M, Oshima M, Albargi O, Huldj CW, Ahlstedt I, Clausen M, et al. Modeling human pancreatic beta cell dedifferentiation. Molecular metabolism. 2018;10:74–86. https://doi.org/10.1016/j.molmet.2018.02.002.

5. Efstr S. Mechanisms of adult human β-cell in vitro dedifferentiation and redifferentiation. Diabetes, obesity & metabolism. 2016;18(Suppl 1):97–101. https://doi.org/10.1111/dobm.12724.

6. Chen H, Zhou W, Ruan Y, Yang L, Xu N, Chen R, et al. Reversal of angiotensin II-induced beta-cell dedifferentiation via inhibition of NF kappaB signaling. Mol Med. 2018;24019–0044.

7. Diedsheim M, Oshima M, Albargi O, Huldj C, Ahlstedt I, Clausen M, et al. Modeling human pancreatic beta cell dedifferentiation. Mol Metab. 2018;10:74–86 Epub 2018 Feb 8.

8. Thakkar U, Trivedi H, Vanikar A, Dave S. Insulin-secreting adipose-derived mesenchymal stromal cells with bone marrow-derived hematopoietic stem cells from autologous and allogenic sources for type 1 diabetes mellitus. Cytotherapy. 2015;17(7):940–7 Epub 2015 Jul 4.

9. Ranjbaran H, Mohammadi JB. Efficacy of mesenchymal stem cell therapy on β2-adrenergic resistance. ACS Nano. 2019;13(2):2450–2461.

10. Hashemin SR, Aliannejad R, Zarrabi M, Soleimani M, Vosough M, Hosseini SE, et al. Mesenchymal stem cells derived from perinatal tissues for treatment of critically ill COVID-19-induced ARDS patients: a case series. Stem Cell Res Ther. 2017;12:200.

11. Su T, Xiao Y, Xiao Y, Guo Q, Li C, Huang Y, et al. Bone marrow mesenchymal stem cells from autologous and allogenic sources for type 1 diabetes mellitus. Cytotherapy. 2015;17(7):940–7 Epub 2015 Jul 4.

12. Zhao K, Hao H, Liu J, Tong C, Cheng Y, Xie Z, et al. Bone marrow-derived mesenchymal stem cells ameliorate chronic high glucose-induced beta-cell injury through modulation of autophagy. Cell Death Dis. 2015;17:230.

13. Wang L, Qin L, Liu H, Liu N, Qiao J, Cui C, et al. Mesenchymal stem cell-derived mesenchymal stromal cells ameliorate oxidative-stress induced islet endothelium apoptosis and functional impairment via Wnt/beta-catenin signaling. Stem Cell Res Ther. 2017;8:107–0640.

14. He Q, Wang L, Zhao R, Yan F, Sha S, Cui C, et al. Mesenchymal stem cell-derived exosomes exert ameliorative effects in type 2 diabetes by improving hepatic glucose and lipid metabolism via enhancing autophagy. Stem Cell Res Ther. 2020;11(1):223. https://doi.org/10.1186/s13287-020-01731-6.

15. Tkach M, Thery C. Communication by extracellular vesicles: where we are and where we need to go. Cell. 2016;164(9):1226–32. https://doi.org/10.1016/j.cell.2016.01.043.

16. Gonzalez-King H, Garcia N, Ontoria-Oviedo I, Ciria M, Montero J, Sepulveda S. Hypoxia inducible factor-1alpha potentiates jagged 1-mediated angiogenesis by mesenchymal cell-derived exosomes. Stem Cells. 2017;35(7):1577–9 Epub 2017 Apr 24.

17. Farnitsova A, Jeremic N, Tyagi S. Exosomes: cell-created drug delivery systems. Mol Cell Biochem. 2019;455(1–2):1–6.

18. Kim HY, Kim TJ, Kang L, Kim YJ, Kang MK, Kim J, et al. Mesenchymal stem cell-derived magnetic extracellular nanovesicles for targeting and treatment of ischemic stroke. Biomaterials. 2020;243:119942. https://doi.org/10.1016/j.biomaterials.2020.119942.

19. Chen J, Chen J, Cheng Y, Fu Y, Zhao H, Tang M, et al. Mesenchymal stem cell-derived exosomes protect beta cells against hypoxia-induced apoptosis via miR-21 by alleviating ER stress and inhibiting p38 MAPK phosphorylation. Stem Cell Res Ther. 2020;11(1):197. https://doi.org/10.1186/s13287-020-01610-0.

20. Li D, Zhang J, Li J. Role of miRNA sponges in hepatocellular carcinoma. Clinica Chimica Acta Int J Clin Chem. 2020;500:10–9.
42. Qian B, Zhou X, Li B, Li B, Liu Z, Wu J, et al. Reduction of pancreatic beta-cell dedifferentiation after gastric bypass surgery in diabetic rats. J Mol Cell Biol. 2014;6(6):531–4 Epub 2014 Oct 31.

43. Fiori J, Shin Y, Kim W, Krzysik-Walker S, Gonzalez-Mariscal I, Carlson O, et al. Resveratrol prevents beta-cell dedifferentiation in nonhuman primates given a high-fat/high-sugar diet. Diabetes. 2013;62(10):3500–13 Epub 2013 Jul 24.

44. Xuan X, Gao F, Ma X, Huang C, Wang Y, Deng H, et al. Activation of ACE2/angiotensin (1-7) attenuates pancreatic beta cell dedifferentiation in a high-fat-diet mouse model. Metabolism. 2018;81:83–96 Epub 2017 Dec 7.

45. Yao X, Wei W, Wang X, Chenglin L, Björklund M, Ouyang H. Stem cell derived exosomes: microRNA therapy for age-related musculoskeletal disorders. Biomaterials. 2019;224:119492. https://doi.org/10.1016/j.biomaterials.2019.119492.

46. Lovis P, Rogglist E, Laybutt D, Gattesco S, Yang J, Widmann C, et al. Alterations in microRNA expression contribute to fatty acid-induced pancreatic beta-cell dysfunction. Diabetes. 2008;57(10):2728–36 Epub 2008 Jul 15.

47. Liu X, Fan B, Szalad A, Jia L, Wang L, Wang X, et al. MicroRNA-146a mimics reduce the peripheral neuropathy in type 2 diabetic mice. Diabetes. 2017;66(12):3111–21 Epub 2017 Sep 12.

48. Menta E, Giuliani A, Matacchione G, Gurau F, Bonfigli A, Romagnoli F, et al. Circulating miR-146a in healthy aging and type 2 diabetes: age- and gender-specific trajectories. Mech Ageing Dev. 2019;180:1–10. https://doi.org/10.1016/j.mad.2019.03.001.

49. Kovacs B, Lumayag S, Cowan C, Xu S. MicroRNAs in early diabetic retinopathy in streptozotocin-induced diabetic rats. Invest Ophthalmol Vis Sci. 2011;52(7):4402–9. https://doi.org/10.1167/iows.10.6879.

50. Rogglist E, Britan A, Gattesco S, Lin-Maqr N, Abderrahmani A, Meda P, et al. Involvement of microRNAs in the cytotoxic effects exerted by proinflammatory cytokines on pancreatic beta-cells. Diabetes. 2010;59(4):978–86. https://doi.org/10.2337/db09-0881.

51. Lindeløv Vestergaard A, Heiner Bang-Berthelsen C, Flyset T, Lucien Stahl J, Christen L. MicroRNAs and histone deacetylase inhibition-mediated protection against inflammatory β-cell damage. 2018;13:e0203713.

52. Forloni M, Dogra SK, Dong Y, Conte D Jr, Ou J, Zhu LJ, et al. miR-146a promotes the initiation and progression of melanoma by activating Notch signaling. eLife. 2014;3:e01460. https://doi.org/10.7554/eLife.01460.

53. Hu ZJ, He JF, Li KJ, Chen J, Xie XR. Decreased microRNA-146a in CD4+ T cells promote ocular inflammation in thyroid-associated ophthalmopathy by targeting NUbM. Eur Review Med Pharmaco Sci. 2017;21(8):1803–9.

54. Hwang WL, Jiang JK, Yang SH, Huang TS, Lai HY, Teng HW, et al. MicroRNA-146a directs the symmetric division of Snail-dominant colorectal cancer stem cells. Nature Cell Biol. 2014;16(3):268–80. https://doi.org/10.1038/ncb2910.

55. Dabernat S, Secrest P, Peuchant E, Moreau-Gaudry F, Dubus P, Sarvetnick N. Lack of beta-catenin in early life induces abnormal glucose homeostasis in mice. Diabetologia. 2009;52(8):1608–17 Epub 2009 Jun 10.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.