MicroRNA-9 modified bone marrow-derived mesenchymal stem cells (BMSCs) repair severe acute pancreatitis (SAP) via inducing angiogenesis in rats

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Abstract

Background: Severe acute pancreatitis (SAP) is an acute abdominal disease characterized by pancreatic necrosis and systemic disease. In a previous study, we showed that bone marrow-derived mesenchymal stem cells (BMSCs) can reduce SAP by secreting microRNA (miR)-9; however, the underlying mechanism remains unclear. The present study investigated the mechanism underlying BMSC-induced pancreatic regeneration.

Methods: BMSCs were isolated, and miR-9 modified/antagonized BMSCs (pri-miR-9-BMSCs/TuD-BMSCs) were generated and injected into SAP rats. The levels of inflammatory cytokines and histopathologic changes were examined using ELISA and H&E staining. Angiogenesis was analyzed by qRT-PCR, western blotting, and immunohistochemistry. Cell function tests, dual luciferase reporter assays, cell co-culture, western blotting, and cell tracing were used to explore the mechanisms underlying miR-9 induced angiogenesis.

Results: Pri-miR-9-BMSCs induced angiogenesis in SAP rats (Ang-1↑, TIE-2↑, and CD31↑) and repaired damaged vascular endothelial cells (VECs) in vitro, promoting angiogenesis (Ang-1↑, TIE-2↑, PI3K↑, AKT↑, p-AKT↑, CD31↑, and CD34↑). Pri-miR-9-BMSCs released miR-9 into VECs or injured pancreatic tissue, targeting the VE-cadherin gene and promoting PI3K/AKT signaling to treat SAP (VE-cadherin↓, β-catenin↓, PI3K↑, p-AKT↑), whereas antagonizing miR-9 in BMSCs did not alleviate or aggravated SAP.

Conclusions: Pri-miR-9-BMSCs can repair injured pancreatic tissue by secreting miR-9 and promoting angiogenesis.
pro-inflammatory cytokines, which alters cellular integrity and increases permeability, leading to microcirculatory disturbances, tissue edema, infiltration of inflammatory cells, and the release of pro-inflammatory cytokines [3, 4]. Therefore, the repair of injured blood vessels contributes to decreasing the local/systemic inflammatory response and improves the local/systemic microcirculation [5]. Injury to blood vessels occurs before the development of AP [3]. Therefore, the development of methods to repair injured blood vessels has become a research hotspot and could be a new target for the treatment of AP. An adequate blood supply provides essential nutrients to pancreatic cells, which is important for supporting metabolism and growth [4, 6]. In addition, the injured pancreas can be repaired in the presence of an adequate blood supply to support the self-renewal of pancreatic cells [4]. Tissue regeneration associated with anti- and pro-angiogenic signaling pathways mainly depends on the formation of new blood vessels, which is mediated by a complex process [7]. The PI3K/AKT signaling pathway, which can promote the proliferation and migration of VECs to trigger angiogenesis, has been investigated extensively [8–10]. Conversely, the VE-cadherin-catenin complex can strongly stabilize endothelial junctions against the migration of VECs, which can inhibit angiogenesis [11–13]. Cellular growth factors also play an important role in inducing angiogenesis by acting on their receptors to start downstream signal transduction and promote the proliferation and migration of VECs [4]. For example, vascular endothelial growth factor (VEGF) and its receptor, VEGFR, can activate the PI3K/AKT pathway to trigger revascularization [12]. Angiopoietin-1 (ang-1), which is highly related to endothelial cell survival, proliferation, and migration, can reduce endothelial permeability and promote the maturation and stability of newly formed blood vessels by interacting with the tyrosine kinase TIE-2 receptor [6, 14].

Mesenchymal stem cells (MSCs) are adult stem cells of low immunogenicity that possess specific properties such as self-renewality, multilineage differentiation, immunosuppression, directed migration, and paracrine functions [15]. Therefore, MSCs are considered as the ideal seed cells for treating human diseases including autoimmune and liver diseases [16]. Bone marrow-derived mesenchymal stem cells (BMSCs) have been investigated extensively. Recent studies including ours demonstrated that infused BMSCs can decrease the local systemic inflammatory response and repair injured pancreatic tissue. Transplanted BMSCs promote the expression of VEGF-A, Ang-1, HGF, and TGF-β in the damaged pancreas and induce angiogenesis [4]. However, the underlying mechanism remains unknown. Despite extensive efforts, necrotic pancreatic tissues cannot be completely repaired in the early stage of SAP. Therefore, exploring the potential of infused MSCs for the treatment of SAP and how to improve their curative effect are important issues that need to be addressed.

MicroRNAs (miRNAs), which are endogenous noncoding RNAs of 18–24 nucleotides, are involved in virus defense, hematopoiesis, organ formation, cell proliferation, inflammatory responses, apoptosis, and lipid metabolism. miRNAs function by binding to the 3’ untranslated region (3’-UTR) of target genes, promoting mRNA degradation or repressing protein translation [17–19]. Recent studies show that miRNAs are useful as biomarker targets for the diagnosis/treatment of AP [18, 19]. In our previous study, we showed that miR-9 expression was negatively correlated with the severity of AP, and injection of miR-9 mimics acts on SAP by targeting the NF-κB1 gene and inhibiting NF-κB signaling. Studies show that miR-9 has the ability to promote the migration of vascular endothelial cells and induce angiogenesis [20–21]. In our previous study, we investigated that BMSCs can repair SAP through miR-9, which could inhibit the inflammatory response [17]. However, we also observed that BMSCs promoted the regeneration of the pancreas. In the present study, we investigated the effect of BMSCs on promoting the repair of necrotized pancreatic tissues and the mechanism by which miR-9-modified BMSCs induce the angiogenesis.

**Methods**

**Materials**

Na-taurocholate (NaT), poly-l-lysine, nitrocellulose membrane, trypan blue, 4′, 6-diamidino-2-phenylindole (DAPI), polybrene, dimethyl sulfoxide (DMSO), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), lipopolysaccharide (LPS), and secondary antibodies were purchased from Sigma-Aldrich (Brooklyn, NY, USA). The apoptosis detection kit was from BD company (Becton, Dickinson and Company, NY, USA). The amylase and lipase activity assay kits were from Biovision (Palo Alto, California, USA). SPION (Fe3O4, 30 nm) were from Dk Nanotechnology Company (Beijing, China). Penicillin, streptomycin, Cell Tracker CM-Dil, TRizol, TRIzol LS Reagent, Lipofectamine 2000 (Lipo2000), and the Histostain-Plus Kit (DAB, Broad Spectrum) were from Invitrogen (Carlsbad, California, USA). RIPA lysis buffer, BCA protein concentration assay kit, phenylmethanesulfonyl fluoride (PMSF, 100 mM), Dulbecco’s modified Eagle’s medium–high/low glucose (DMEM-H/LG), 0.25% Trypsin-EDTA and fetal bovine serum (FBS) were from Gibico (Middleton, WI, USA). The Prussian Blue staining kit were from Beyotime Biotechnology (Nantong, Jiangsu Province, China). Agarose was from Biowest (Spain). Antibodies directed against Glyceraldehyde-phosphate dehydrogenase (GAPDH), VEGFA, angiopoietin-1(Ang-1) and
TIE-2 are from ProteinTech (Wuhan, Hubei Province, China), β-catenin, VE-cadherin, PI3K, AKT, and p-AKT from CST (Danvers, MA, USA), CXCR4 from Abcam (Cambridge, MA, USA), PECAM-1 (CD31) from Santa Cruz Biotechnology (Santa Cruz, CA, USA). IL-1β, IL-4, IL-6, IL-10, TNF-α, and TGF-β enzyme-linked immunosorbent assays (ELISAs) kits were purchased from R&D Systems (Minneapolis, MN, USA). Restriction endonuclease, competent *Escherichia coli* (DH5α), Taq enzyme, PrimeScript Reverse Transcriptase Reagent Kit and Primer STAR Max DNA Polymerase, MutanBEST Kit, and T4 polynucleotide kinase are from Takara Biotechnology (Dalian, Liaoning Province, China). DNA purification kit, Dual Luciferase Reporter Assay System, was from Promega corporation (Beijing, China), TIANprep Mini Plasmid Kit and TIANgel Midi Purification Kit are from Tiangen Biotechnology company (Beijing, China).

**Cell culture**

Bone marrow-derived mesenchymal stem cells (BMSCs) were isolated from the bone of 3–4 weeks of Sprague-Dawley (SD) rats and cultured in DMEM-LG complete medium as previously described [4, 17]. HEK-293T cells (human embryonic kidney-293 cells expressing the large T-antigen of simian virus 40) were purchased from the cell bank of Chinese Academy of Sciences and cultured in DMEM-HG supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Vascular endothelial cells (VECs) were isolated from the aortimal aortic of SD rats as previously described [22] and cultured in DMEM-HG complete medium. Finally, these cells were digested and passaged when reaching 80% of confluence.

**Construction of cell lines of pri-miR-9-BMSCs and TuD-BMSCs**

The establishment of cell lines of pri-miR-9-BMSCs and TuD-BMSCs was performed as previously described [17]. In brief, rat genomic DNA was extracted using a DNA purification kit, and a 368-bp DNA fragment containing the miR-9-1 sequence (NC_005101.4) was amplified and inserted into the PCDH plasmid to form the recombinant vector of pri-miR-9-PCDH. The lentivirus encoding miR-9 was produced by a lentivirus packaging system (System Biosciences, CA, and USA) as previously described [17]. The anti-miR-9 plasmid was constructed by applying the RNA tough decoy (TuD) technique as previously described [23]. The lentivirus encoding TuD was also produced by a lentivirus packaging system. BMSCs were infected with pri-miR-9 or empty- and TuD-lentivirus to establish the cell lines of pri-miR-9-BMSCs, empty virus BMSCs (EV-BMSCs), and TuD-BMSCs.

**Transfection of BMSCs with Cy3-miR-9a-5p mimics and co-culture with VECs**

BMSCs were transfected with Cy3-miR-9a-5p mimics or miR-9a-5p control at a final concentration of 100 nM using Lipo2000 as previously described [17]. After transfection, they were digested, added into the upper chambers of six cluster plates (Costar Transwell™ Permeable Supports, 0.4-µm), and co-cultured with VECs. The mRNAs and proteins were then extracted using the TRIzol reagent and RIPA lysis buffer at 48 and 72 h, respectively. The expression of genes of interest was verified by general PCR (gPCR) and western blotting. The experiments were repeated three times.

**Cell apoptosis and proliferation**

The cell apoptosis was performed by the apoptosis detection kit as previously described [16]. In brief, BMSCs transfected with miR-9 mimic or miR-9 control was treated with or without LPS (1 µg/ml) for 24 h. Then, cells were preincubated with annexin V at room temperature in the dark for 15 min, followed by the addition of propidium iodide (PI). Finally, the percentage of apoptotic cells was analyzed by flow cytometry (BD Biosciences). To evaluate the effect of miR-9 on VECs, the cell proliferation test (MTT) was also conducted as previously described [4]. First, VECs were transfected with miR-9 mimic or miR-9 control. Then, MTT solution (20 µl of 5 mg/ml) was added for 6 days (12 h, 36 h, 60 h, 84 h, 108 h, and 132 h). Third, the medium was removed and 150 µl of DMSO was added to each well, which was shaken slowly for 10 min. Finally, the absorbance was measured by an

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**Table 1** The sequence of primers

| Gene   | Forward (5′-3′) | Reverse (5′-3′) |
|--------|----------------|-----------------|
| TIE-2  | CTGGGCATGACATGTGCAG | GCAAATGATGGTCCTCTACAAGG |
| Ang-1  | CAAGGGTGTACTGAGG | CCGATGCTTCAGATGGTC |
| GAPDH  | CGGGCGGATCTGACATGC | CTTGCTCTCAGTATCCTTGC |
| Primers | miR-9 (5′-3′) | U6 (5′-3′) |
| Reverse transcription | GTGCTATCCAGTCAGG | AGGCTACGAGTTCGGGCT |
| Forward | GCTCTTTCGATTCATCAGT | CTTGCTTCCAGGCGACCTT |
| Reverse | GTGCAAGGTCGGAGGT | AGGCTACGAGTTCGGGCT |

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Fig. 1 (See legend on next page.)
ELISA plate reader at 490 nm. This experiment was repeated three times.

**Cell migration and angiogenesis**

VECs transfected with miR-9 mimic or miR-9 control were added to the upper chamber of the Transwell apparatus. After incubation for 12 h, the upper chamber was fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet (sigma) for 10 min in the dark and photographed by the phase-contrast microscopy. Finally, the crystal violet was dissolved in 300 μl of 33% acetic acid (sigma) and the absorbance of the solution was measured by an ELISA plate reader (Gene Company Limited, HK, China). To investigate the effect of the miR-9 on the angiogenic activity of VECs in vitro, we performed a tube formation assay. Ninety-six-well culture dishes were coated with 50 μl of matrigel matrix (BD company) and incubated for 30 min at 37 °C. VECs transfected with miR-9 mimic or miR-9 control were seeded onto the solidified gels at a density of 2 × 10⁶ cells/well in 50 μl of culture medium. After incubation for 12 h, the total tube-like structures were photographed by phase-contrast microscopy (×100) and counted. These two experiments were repeated three times.

**Animal models**

Male SD rats weighing 200–250 g (n = 100) were purchased from Shanghai Laboratory Animal Co. Ltd. (Shanghai, China) and fed in a suitable environment with 25 °C and 12-h dark/light cycle, given free access to water and food. The AP models were induced by the intraperitoneal injection of caerulein (100 μg/kg) for three times or the retrograde injection of 3%NaT (1 ml/kg) as previously described [15, 16]. All the procedures conform to the Ethics of Yijishan Hospital, affiliated to Wannan Medical School (Wuhu City, Anhui Province, China) and the Ethics of Shanghai Tenth People’s Hospital, affiliated to Tongji University (Shanghai, China).

**Cell transplantation, animal grouping, and sample preparation**

Rats were randomly injected with pri-miR-9-BMSCs, EV-BMSCs (EV-BMSCs), TuD-BMSCs, or BMSCs (1 × 10⁷ cells/kg) by the tail vein at postoperative day 1 as previously described [15, 16] and divided into NC (n = 6), Sham (n = 6), SAP (n = 6), SAP + PBS (PBS treatment) (n = 6), BMSCs (n = 6), pri-miR-9-BMSCs (n = 6), EV-BMSCs (n = 6), and TuD-BMSCs (n = 6) groups. To investigate the relationship between miR-9 and AP, several AP models were established as follows: NC (n = 3), Sham (n = 3), caerulein (n = 3), 1% NaT (n = 3), and 3% NaT (n = 3). Rats were humanely killed after BMSC treatment. The serum was collected by centrifugation at 8000×g at 4 °C for 20 min and stored at −80 °C. The tissues were obtained by surgical vehicles and stored in liquid nitrogen or at −80 °C or fixed in 4% paraformaldehyde.

**Hematoxylin–eosin (H&E) staining, ELISAs, and the levels of amylase and lipase**

The H&E staining of paraffin-embedded pancreatic tissues was performed for assessing the severity of AP as previously described [4, 17]. The levels of serum IL-1β, IL-4, IL-6, IL-10, TNF-α, and TGF-β were detected by ELISA kit as previously described. The activities of serum amylase and lipase were assayed by the amylase and lipase assay kit as previously described [16].

**General PCR (gPCR) and quantitative real-time PCR (qRT-PCR)**

Total RNA was extracted using TRIzol or TRIzolLS Reagent. cDNA was synthesized using a PrimeScript Reverse Transcriptase Reagent Kit. The expression of genes of interest was detected by gPCR or qRT-PCR as previously described [17]. GAPDH and U6 were used as endogenous controls. The sequences of the primers are listed in Table 1. Each measurement was performed in three repeats.

**Immunoblotting and immunohistochemistry**

The immunoblotting (western blot) procedure used in the study was described previously [17]. In brief, total proteins were extracted using RIPA lysis buffer with PMSF (1:100) and protease inhibitor cocktail tablets (Roche Applied Science, Shanghai, China), and the BCA method was used for the protein quantification. Proteins...
Fig. 2 (See legend on next page.)
were transferred to a nitrocellulose membrane, which was detected by the Odyssey 3.0 analysis software (LI-COR Biotechnology, Nebraska, USA) after incubation with primary and secondary antibodies. Immunohistochemistry was performed as previously described [17]. The experiments were repeated three times.

**MiRNAs target prediction**

The prediction of miRNA target genes was performed by the algorithms of TargetScan [24], PicTar [24], microRNAorg [25], and miRWalk Targets [26]. The results of predicted targets were intersected by using MatchMiner [27], showing that the VE-cadherin gene was the target gene of miR-9.

**Dual luciferase reporter assays**

A fragment of VE-cadherin 3′-UTR (222 bp) containing the putative miR-9 binding site was amplified by Primer STAR Max DNA Polymerase using the following primers: 5′-GAAGCCAGA AAAACGGGACCCCGG-3′ and 5′-GC CACGGGATGGAAGTGACTTTG-3′, and cloned into psiCHECK-2 vector (Promega, Beijing, USA). Finally, the recombinant psiCHECK-2-VE-cadherin 3′-UTR (wtUTR) plasmid was identified by the sequence analysis. Besides, a mutant VE-cadherin 3′-UTR fragment (222 bp) with five base mutation (CAAGCC AGCGGC ACGGC CCTGGA-3′ and 5′-GG CACGGGGACCCCGGACCCCGG-3′) and cloned into psiCHECK-2 vector (Promega, Beijing, USA). The results of predicted targets were intersected by using MatchMiner [27], showing that the VE-cadherin gene was the target gene of miR-9.

**Tracking of cy3-miR-9a-5p in vivo after the transplantation of EV-BMSCs transfected with Cy3-miR-9a-5p**

The liver, heart, spleen, lung, pancreas, kidney, and duodenum were collected on day 3 after the transplantation of EV-BMSCs transfected with Cy3-miR-9a-5p and fixed in 4% paraformaldehyde for 24 h. Then, the organs were dehydrated in a 30% sucrose solution and embedded using the Tissue-Tek O.C.T. Compound (SAKURA, USA) and solidified into a tissue block at −80 °C. The tissue block was cut into 5 μm frozen sections and observed under a fluorescence microscope. The red particles were counted in five randomly selected fields at ×200 magnification. SPION was also used for labeling BMSCs to trace their distribution in vivo as previously described [4].

**Image processing and statistical analysis**

Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA), Image-Pro Plus version 6.0 (Media Cybernetics, USA), and ImageJ (National Institutes of Health, USA) were applied for image typesetting, analysis, and processing. GraphPad Prism 5.1 (GraphPad Co., USA) was used for mapping. The statistical analyses were conducted by SPSS 18.0 statistical software (Chicago, IL). Experimental data are shown as means ± standard deviations (SD) and compared with Student’s or a paired t test or one-way ANOVA. A value of P < 0.05 was deemed to indicate significant differences.

**Results**

Infused BMSCs reduce SAP and inhibit systemic inflammatory responses in a miR-9-dependent manner

A rat model of SAP was established, and BMSCs were isolated and cultured as previously described [4]. Then, BMSCs were infused into SAP rats and their effect was investigated. Detection of the expression of miR-9 in
pancreatic tissues showed that miR-9 levels were lower in the SAP group and higher in the BMSC group. miR-9 modified/antagonized BMSCs (pri-miR-9-BMSCs/TUdBMSCs) and empty virus BMSCs (EV-BMSCs) were injected into SAP rats as previously described [4, 15, 17]. The results showed that pri-miR-9-BMSCs markedly promoted the repair of damaged pancreatic tissues (pancreatic edema↓, infiltration↓, hemorrhage↓, and necrosis↓), decreased the levels of amylase and lipase, and inhibited systemic inflammatory responses (TNF-α↓, IL-1β↓, IL-6↓,

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**Fig. 3** (a, b) The distributions in vivo of CM-Dil- and SPION-labeled BMSCs were observed by fluorescence microscopy and Prussian Blue staining. The number of cells migrating to the damaged pancreas was smaller than that of cells migrating to the liver, spleen and lung. Data are shown as the mean ± SD. ***p < 0.001, compared with liver, ###p < 0.001, compared with spleen, @@@p < 0.001, compared with lung by using two-tailed t test
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Transplantation was difficult to explain (Fig. 3). On repairing injured pancreatic tissues on day 3 after cell transplantation. Therefore, the direct effect of BMSCs migrating to the lung, spleen, and liver at 3 days after to the pancreas was small and lower than that of cells injured pancreas; however, the number of cells migrating showed that transplanted BMSCs could migrate to the other organs, which can promote the proliferation, migration, and angiogenesis of vascular endothelial cells.

Few BMSCs could move to the damaged pancreatic tissue
To observe the migration of the infused cells in SAP rats, CM-Dil and superparamagnetic iron oxide nanoparticles (SPION) were used for labeling BMSCs, and the number of red and blue particles in pancreatic tissues was counted by fluorescence microscopy or iron staining as previously described [4, 17]. The results showed that transplanted BMSCs could migrate to the injured pancreas; however, the number of cells migrating to the pancreas was small and lower than that of cells migrating to the lung, spleen, and liver at 3 days after transplantation. Therefore, the direct effect of BMSCs on repairing injured pancreatic tissues on day 3 after cell transplantation was difficult to explain (Fig. 3).

BMSCs deliver exogenous miR-9 into the pancreas and other organs, which can promote the proliferation, migration, and angiogenesis of vascular endothelial cells
To trace the distribution of transplanted BMSCs in vivo, synthetic Cy3-miR-9a-5p (red) was transplanted into GFP-BMSCs (green) and these cells were infused into SAP rats by the tail veins as previously described. The results showed that Cy3-miR-9a-5p was released from GFP-BMSCs into the damaged pancreas and other organs; however, a higher number of infused BMSCs migrated to the lung, liver, and spleen than to the pancreas (Fig. 4a, b). miR-9 inhibited apoptosis and promoted the proliferation, migration, and angiogenesis of VECs (Fig. 5).

**Fig. 4** miR-9, released from BMSCs into VECs, can target the VE-cadherin gene and induce the expression of angiogenic genes in VECs. **a** miR-9 in pancreatic tissues was downregulated in the PBS + SAP group compared with the NC group, whereas it was significantly upregulated by pri-miR-9-BMSCs, compared with SAP, PBS + PBS, BMSCs, EV-BMSCs, and TuD-BMSCs. Data are shown as the mean ± SD for at least three separate experiments. ^p < 0.05, compared with NC; ^p < 0.01, compared with NC. **p<0.01, compared with SAP. ^6p < 0.001, compared with PBS treatment. ^p < 0.05, compared with BMSCs. ^p < 0.05, compared with EV-BMSCs. ^p < 0.05, compared with TuD-BMSCs by using two-tailed t test. **b** GFP-BMSCs could deliver exogenous Cy3-miR-9a-5p to the liver, spleen, lung, and pancreas, and the number was higher in the liver and spleen. **c** Eight paired bars between miR-9 and VE-cadherin and the structure of the wild-type VE-cadherin 3′-UTR (wtUTR) or mutant VE-cadherin 3′-UTR (mutUTR). (mutUTR) were cloned into the psiCHECK-2 plasmid to produce the recombinant vectors, wtUTR psiCHECK-2 and mutUTR psiCHECK-2, harboring the predicted binding sites of miR-9. **d** The activity of firefly luciferase was significantly decreased by miR-9 and rescued by mutant of VE-cadherin 3′-UTR. **e–g** Co-culture of VECs and BMSCs transfected with cy3-miR-9 showed that BMSCs secreted cy3-miR-9 into VECs and inhibited the expression of VE-cadherin and β-catenin and upregulated Ang-1, CXCR4, TIE-2 and p-AKT. p < 0.05, ^p < 0.01, and **p < 0.001, compared with NC group, ^p < 0.05, **p < 0.01, and ***p < 0.001, compared with miR-9 con by using paired t test. VECs, vascular endothelial cells; BMSCs, bone marrow-derived mesenchymal stem cells; GFP, green fluorescent protein; gPCR, general PCR; qRT-PCR, quantitative real-time PCR; SAP, severe acute pancreatitis; NC, normal control; miR-9 con, miR-9 mimic control.

**VE-cadherin is a target of miR-9**
The VE-cadherin transcript and the miR-9a-5p seed sequence have eight pairs of bases at both putative target sites. VE-cadherin and β-catenin were markedly downregulated in VECs after miR-9a-5p transient overexpression. To determine whether miR-9a-5p can target the VE-cadherin gene, we constructed luciferase reporter vectors for VE-cadherin wild-type (wtUTR) or mutant 3′-UTR (mutUTR) harboring the predicted binding sites for miR-9a-5p. The results showed that the relative activity of firefly luciferase in 293T cells was markedly decreased after transfection of miR-9a-5p mimics. The luciferase reporter repression was rescued by mutating the VE-cadherin 3′-UTR. Taken together, these results indicated that VE-cadherin was the target gene of miR-9a-5p (Fig. 4c–g).
**Fig. 5** (See legend on next page.)

**a**

LPS

- LPS
- LPS+miR-9 mimic
- LPS+miR-9 control

**b**

Apoptosis (%) for:

- LPS
- LPS+miR-9 mimic
- LPS+miR-9 control

**c**

The curve of EC growth:

- VECs+miR-9
- VECs+miR-9 control

**d**

VECs+miR-9 control

VECs+miR-9 mimic

**e**

OD Values (OD=573 nm) for:

- miR-9 control
- miR-9 mimic

**f**

VECs+miR-9 control

VECs+miR-9 mimic

**g**

The number of tube formation for:

- miR-9 control
- miR-9 mimic

(See legend on next page.)
Discussion

The incidence of AP is increasing gradually, and the underlying pathogenetic mechanism remains undefined [28]. Most AP cases can be cured by conventional medical treatment. However, 10 to 20% of AP cases can evolve into SAP, which is a systemic disease characterized by pancreatic necrosis, systemic inflammatory responses, and multiple organ dysfunction [1, 2, 28]. Preventing the occurrence of SAP is currently not possible, and the disease process is not completely understood. Repairing injured pancreatic tissues thoroughly cannot be achieved, and the mortality of SAP remains at approximately 40% [2, 4, 28]. The morbidity of SAP also affects the quality of life of patients, who often require pancreatic enzymes or insulin injections to support their lives. The high mortality and morbidity not only makes patient suffering and decreased quality of life [33, 34]. Therefore, many researchers have started to focus on pancreatic regeneration, which can eradicate pancreatic diseases and alleviate pain. The process of regeneration must be inseparable from angiogenesis, which is essential for tissue regeneration [35, 36].

BMSCs have the potential for secreting cellular growth factors and promoting angiogenesis, although the underlying mechanism remains unknown [4]. In the present study, we explored the possible mechanism using genetic engineering methods and by constructing miR-9 modified/antagonizing BMSCs (pri-miR-9-BMSCs and TuD-BMSCs). The results showed that pri-miR-9-BMSCs promoted angiogenesis more effectively than BMSCs and TuD-BMSCs. The expression levels of Ang-1, TIE-2, CXCR4, and p-AKT in pri-miR-9-BMSCs were also the highest and the lowest in TuD-BMSCs. These results showed that angiogenesis was regulated by miR-9 in BMSC therapy for SAP.

Further, we demonstrated that the angiogenesis was inhibited in injured pancreatic tissues, showing the correlation with the severity of pancreatitis and miR-9 was positively correlated with the angiogenesis. Besides, transfection of miR-9 mimics into VECs showed that miR-9 significantly inhibited the expression of angiogenesis-related genes (VEGF, Ang-1, TIE-2, CXCR4, and p-AKT). These data demonstrated that miR-9 was involved in the process of angiogenesis and could promote its occurrence.

To further reveal the mechanism underlying the effect of miR-9 on promoting angiogenesis, we predicted the target gene of miR-9 using online software and found that VE-cadherin is a target gene of miR-9. To confirm that miR-9 can repress the expression of VE-cadherin by directly binding to the VE-cadherin 3′-UTR, we performed a dual luciferase reporter assay and detected the expression of the VE-cadherin gene in VECs after co-culture with BMSCs transfected with miR-9 mimics. The results showed that miR-9 could reduce the activity
Fig. 6 (See legend on next page.)
BMSCs migrate to injured pancreas (Additional file 1).

It was speculated that miR-9-induced angiogenesis might help iterative days 5–7, and the result showed that SPION-labeled BMSCs gradually migrated to injured pancreas and peaked on postoperative day 10. The possible reason for the sharp decline is that those BMSCs might have differentiated into pancreatic cells and regenerate necrotized pancreatic tissue.

In a word, the process of BMSCs promoting pancreatic regeneration is very complex, and the mechanism is difficult to understand. At present, most of the studies on pancreatic regeneration mainly focus on the regeneration of insulin-secreting β-cells rather than the regenerative process of digestive enzyme-producing acinar cells [33]. To our knowledge, little is known about the regeneration of acinar cells. In this study, we revealed that infused BMSCs could induce the angiogenesis at day 3 after cell transplantation by secreting miR-9. Combining with the previous study, we found that miR-9 released by BMSCs could induce angiogenesis, which could help more BMSCs migrate to the injured pancreas and promote the regeneration of necrotized pancreatic tissue.

Conclusions

miR-9 is an important protective factor in SAP, and BMSCs can repair SAP through miR-9, which targets the VE-cadherin gene, modulates β-catenin signaling, and induces angiogenesis. These findings suggest the potential of BMSCs for the treatment of SAP and identify a new therapeutic target.

Additional file

Additional file 1: The possible mechanisms of speculation on BMSC repairing necrotized pancreatic tissue. (JPG 17 kb)

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Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study. If you want, please contact author for data requests.
Authors’ contributions
DQ, GS and ZM performed this experiment. XW and ZS designed the study and wrote the main manuscript. GW and MH collected the data and analyzed the results. LJ checked the data and prepared the pictures. All authors read and approved the final manuscript.

Ethics approval
All animal experiments are approved by the Ethics committee of Yiyishan Hospital, affiliated to Wannan Medical College (Wuhu City, Anhui Province, China) and the Ethics of Shanghai Tenth People’s Hospital, affiliated to Tongji University (Shanghai, China).

Consent for publication
All authors have reviewed the manuscript and approved the publication.

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

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