Thrombin-activated platelet rich plasma (PRP) enhanced osteogenic differentiation of bone marrow mesenchymal stem cells by activating autophagy through miR-140-3p/SPRED2 axis

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Research

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Abstract

Background Mesenchymal stem cells transplantation gradually become a potential treatment for bone defect in clinic practice. This study aimed to investigate the molecular mechanism of PRP and autophagy for osteogenic differentiation in bone marrow mesenchymal stem cells (BMSCs).

Methods Thrombin activated PRP was prepared and the BMSCs were treated with activated PRP with different concentration and transfected with miR-140-3p vector (mimics or inhibitor), si-SPRED2 or co-transfected with miR-140-3p inhibitor and si-SPRED2, respectively. qRT-PCR and Western blotting were used to determine the mRNA expression and protein expression. A luciferase reporter assay was conducted to identified the targeting relationship between iR-140-3p and SPRED2 Subsequently, cell proliferation was detected by MTT and ALP activity was also determined. Alizarin red staining was used for the evaluating the formation of calcium nodules.

Results MiR-140-3p expression was found to be inhibited by PRP in a dose-dependent manner, besides, cell proliferation, ALP activity, the expression of COL-I, OPN, Runx2 and OCN, and the formation of calcium nodules related to osteogenic differentiation were enhanced by PRP. Subsequently, we found that PRP activated autophagy and up-regulated SPRED2 expression in BMSCs through suppressing miR-140-3p expression. Moreover, we confirmed that miR-140-3p targeted SPRED2 and negatively regulation its expression. Finally, the findings showed that inhibition of miR-140-3p enhanced cell proliferation, osteogenic differentiation and autophagy of BMSCs by negatively regulating SPRED2 expression.

Conclusion Thrombin activated PRP accelerated osteogenic differentiation of BMSCs by activating autophagy through miR-140-3p/SPRED2 axis.

Introduction

In order to enhance bone regeneration, various approaches, including bone transport and distraction osteogenesis, have been conducted for the repair of bone defect in the last decades [1]. However, these therapies might lead to donor site injury, infection, deformity, surgical risks and even healthy bone loss [2]. Therefore, mesenchymal stem cells transplantation gradually become a potential treatment for bone defect in clinic practice.

Numerous studies revealed that bone marrow mesenchymal stem cells (BMSCs) have the potential to differentiate into osteoblasts, myoblasts, adipocytes and so on under different conditions, representing an alternative reparative cell type [3]. And the osteogenesis of BMSCs is always considered as a potential therapeutic strategy for bone regeneration. However, the molecular mechanisms for differential of BMSCs are still unclear.

In addition to stem cell therapy, platelet rich plasma (PRP) has also been widely applied in various fields, especially bone defects in maxillofacial region and oral cavity [4]. Moreover, PRP at different concentrations showed different potentials to induce chondrogenic differentiation, migration, and

proliferation of mesenchymal progenitor cells [5]. Recently, more and more researches show that PRP can be used in the field of bone regeneration, and it has been involved widely in orthopedic surgery [6]. It was found PRP could be used in treatment of chronic lateral elbow epicondylitis, mandibular degree II furcation defects and tendinopathy [7–9]. And the application of PRP is considered as a potential therapeutic strategy in bone repair in the future[10]. However, the underlying molecular mechanisms for PRP in osteogenic differentiation are still unknown. Interesting, PRP was reported to activate autophagy in osteoblast precursor 3T3-L1 [11]. Thus, we wondered the relationship between PRP and autophagy in osteogenic differentiation in BMSCs.

In the present study, we investigated the molecular mechanism of PRP and autophagy in BMSCs. We for the first time demonstrated that thrombin-activated PRP enhanced osteogenic differentiation of BMSCs by activating autophagy through miR-140-3p/SPRED2 axis. This study might provide more research basis and might give novel study targets for PRP in clinical treatment of bone regeneration.

Materials And Methods

Preparation of Thrombin Activated Platelet-Rich Plasma(PRP)

For the preparation of PRP, human blood was collected from 8 donors (150 mL of venous blood each) and mixed with 10% acid-citrate-dextrose as an anticoagulant. The whole blood was centrifuged for 25 min and PRP was extracted from the buffycoat into an empty sterile syringe. For the activation, bovine thrombin was added to PRP at 1:5(vol/vol) and incubated for 30 minutes at 37 °C. Then the sample was centrifuged at 4000 rpm for 5 min, and the supernatant was collected and mixed with serum-free DMEM solution to prepare PRP at a concentration of 5%, 10% and 20%.

Cell Culture and Transfection

The human bone marrow stem cells (BMSCs) were obtained from ATCC (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle Medium (DMEM) including 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were incubated in a humidified atmosphere at 37 °C with 5% CO₂. BMSCs were treated with activated PRP at different concentration and transfected with miR-140-3p vector (mimics or inhibitor), si-SPRED2 or co-transfected with miR-140-3p inhibitor and si-SPRED2, respectively.

MTT Assay

The cell viability was detected by MTT assay at 0 h, 24 h, 48 h, 72 h and 96 h after the treatment. BMSCs were planted into 96-well microplates with the density of 5×10^3 and incubated for 48 h. subsequently, 10 µL of 5 mg/mL MTT solution was added to each well and incubated for another 4 h at 37 °C. The supernatant was discarded and replaced with 150 µl DMSO. Finally, the absorbance was measured was measured at 490 nm by using a microplate reader (DNM-9602; Pe rlong, Beijing, China). Each experiment was performed in triplicate and repeated at least three times.

Alizarin Red Staining and Alkaline Phosphatase (ALP) Activity

BMSCs were fixed with 4% paraformaldehyde and incubated with 1% alizarin red S solution (Beyotime, Shanghai, China) for 10 min at room temperature. Excess stain was eliminated using PBS washing. Calcium-bound stain was collected with 100 mM CPC and absorbance value was measured at 405 nm by a microplate reader.

For the detection of ALP activity, BMSCs were cultured in 24-well plates at a cell density of 1×10^4 for 7 days, washed with PBS and fixed with 4% paraformaldehyde. Protein levels were determined by a BCA protein assay kit and the ALP activity was measured by using a ALP detection kit (Wako Chemical, Richmond, VA).

Reverse Transcription-Quantitative Polymerase Chain Reaction (qRT-PCR)

In brief, Total RNA was extracted from BMSCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). For the detection of miRNA expression, RNA was firstly converted into cDNA by using a iScriptTM cDNA Synthesis Kit (Bio-Rad, Reinach, Switzerland). A SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a ABI Prism 7300 (Applied Biosystems) was used for quantification. For the detection of mRNA expression, DyNamoTM cDNA synthesis kit (Fermentas) was used for the reverse transcription. PCR reaction and analysis was performed on an ABI Prism 7500 sequence detection system (PE Applied Biosystems) with a DyNamo SYBR1 Green qPCR kit (Finnzymes, Espoo, Finland). U6 and GAPDH were used as internal controls for miRNA and mRNA, respectively. The following primers were used in PCRs: F 5'-GCTTGGTGGGCTTCTGGT-3' and R 5'-CCCGGTATCCTGTCCTGTCCGTGGT-3' for miR-140-3p; F 5'-CTCATCCATGGTGAACGACAGAA-3' and R 5'-TGTCAAAGGCTCGGGCATC-3' for SPRED2; F 5'-GAGAGCATGACCGATGGAT-3' and R 5'-ATGTTTTGGTGGTTCAGGAGG-3' for COL-1; F 5'-GTGCAGCCTHGTGTCCAAG-3' and R 5'-GTCAGCCAACTCGTCACAGT-3' for OCN; F 5'-CTGAACGCGCCTTCTGATTG-3' and R 5'-ACATCGGAATGCTCATTGCTCT-3' for OPN; F 5'-GGAGTGGACGAGGCAAGAGTTT-3' and 5'-AGCTTCTGTCTGTGCCTTCTGG-3' for Runx2; F 5'-TGCGGGTGCTCGCTTCGGCAGC-3' and R 5'-CCAGTGCAGGGTCCGAGGT-3' for U6; F 5'-ACAGAGCCTCGCCTTTGC-3' and R 5'-GAGGCGTACAGGGATAGCAC-3' for β -actin. The relative expression levels of miRNA or mRNA was calculated by the $2^{-\Delta\Delta C_q}$ method.

Western Blotting

Protein was extracted by RIPA lysis buffer (Thermo Scientific, U.S.A.) from BMSCs and determined using a BCA Protein Assay Kit (ComWin Biotech, Beijing, China). Equal amount of proteins was loaded on 10% SDS-PAGE gels, transferred to PVDF membranes and blocked with 5% non-fat milk at room temperature for 1 h. Afterwards, membranes were subjected to immunoblotting with primary antibodies at 4°C overnight, followed by horseradish peroxidase-conjugated secondary antibodies at 37°C for 1 h. The primary antibodies were listed as follows: SPRED2(ab153700, 1/500)), Beclin1 (ab207612, 1/2000) and LC3 I/II (ab62721, 1/1000) (all from Abcam, Cambridge, MA, U.S.A). β -actin was used as a control. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA) and quantified using AlphaEase FC software.

Luciferase Reporter Assay

Two types of 3'-UTRs (WT and MUT) of SPRED2 sequences containing the binding sequence of miR-140-3p were amplified and inserted into the p-MIR-report plasmid (Ambion, Austin, TX, USA). Subsequently, BMSCs were transfected with miR-140-3p vector (mimics or inhibitor) and SPRED2 luciferase reporter plasmid (WT- SPRED2 or MUT- SPRED2) or NC (mimics or inhibitor) using Lipofectamine 2000 (GeneChem, Shanghai, China). The luciferase activity was detected using luciferase assay kits (Promega, Madison, Wisconsin, U.S.A.) after the transfection of 48 h. Luciferase activity of cells was normalized to Renilla luciferase activity.

Statistical Analysis

All calculations were performed using SPSS 18.0. The continuous data was expressed as mean $\pm$ SD. Comparison between two groups was made using the Student t-test, while, comparison among three or more groups was performed using one-way analysis of variance (ANOVA) following by Turkey analysis. The comparison for rates was made by Chi square test. The differences were considered statistically significant when $P < 0.05$.

Results

PRP promotes the proliferation and osteogenic differentiation of human BMSCs by miR-140-3p inhibition or autophagy activation

BMSCs were transfected miR-140-3p mimics and NC mimics, respectively. QRT-PCR result confirm miR-140-3p was dramatically up-regulated by the transfection with miR-140-3p mimics (Fig. 1A). Subsequently, cells were treated with thrombin-activated PRP at a concentration of 5%, 10% and 20%, respectively. MiR-140-3p was down-regulated in cells treated with PRP in a dose-dependent manner (Fig. 1B). Cell proliferation detected by MTT assay showed that thrombin-activated PRP notably enhanced cell viability in a dose-dependent manner but up-regulated miR-140-3p or inhibited autophagy reversed the effect of activated PRP on cell proliferation (Fig. 1C). ALP activity was also found to be increased by activated PRP in a dose-dependent manner that was reversed by miR-140-3p mimics or 3-MA (Fig. 1D). Besides, osteogenic differentiation related mRNA expression of COL-I, OPN, Runx2 and OCN was significantly elevated by activated PRP in a dose-dependent manner but down-regulated by overexpressed miR-140-3p or inhibited autophagy (Fig. 1E). Alizarin red staining illustrated that thrombin-activated PRP obviously accelerated formation of calcium nodules in a dose-dependent manner, which could be reversed by overexpressed miR-140-3p or inhibited autophagy (Fig. 1F). All the results indicated that PRP promotes cell proliferation and osteogenic differentiation of human BMSCs by miR-140-3p inhibition or autophagy activation.

PRP activates autophagy and up-regulated SPRED2 expression of human BMSCs through suppressing miR-140-3p expression

We further investigated the regulation of miR-140-3p on autophagy and SPRED2 expression in BMSCs treated with thrombin-activated PRP. As shown in Fig. 2A, the expression of SPRED2 was up-regulated by the treatment of PRP, however, it was reversed by overexpressed miR-140-3p or autophagy inhibition.

Western blotting result also showed that the protein level of Beclin1 and SPRED2, and the conversion rate of LC3 I to LC3 II was significantly elevated by PRP in a dose-manner but reversed by miR-140-3p mimics or autophagy inhibitor (Fig. 2B). The above findings suggested that PRP enhanced autophagy and up-regulated SPRED2 expression of human BMSCs through suppressing miR-140-3p expression.

MiR-140-3p negatively regulated SPRED2 expression

Next, we explored the regulation relationship between miR-140-3p and SPRED2. Based on bioinformatics analysis, miR-140-3p could directly bind to the SPRED2 3'-UTR (Fig. 3A). Luciferase reporter assay revealed that fluorescence intensity of WT-SPRED2 was significantly decreased by miR-140-3p mimics but increased by miR-140-3p inhibitor, however, fluorescence intensity of MUT-SPRED2 was affected by neither of them (Fig. 3B). QRT-PCR and western blotting detection also confirmed that SPRED2 expression was obviously suppressed by miR-140-3p mimics but enhanced by miR-140-3p inhibitor (Fig. 3C-D). these results demonstrated that miR-140-3p targeted SPRED2 and negatively regulation its expression.

Inhibition of miR-140-3p enhances cell proliferation and osteogenic differentiation of BMSCs by negatively regulating SPRED2 expression

We further studied the molecular mechanism of miR-140-3p for cell proliferation and osteogenic differentiation of BMSCs. Firstly, cells were transfected with si-SPRED2 or miR-140-3p inhibitor, respectively. Both qRT-PCR and western blotting result showed that SPRED2 and miR-140-3p was knockdown successful (Fig. 4A). As shown in Fig. 4B, cell proliferation was suppressed by SPRED2 knockdown. And miR-140-3p inhibitor obviously increased cell proliferation, which was reversed by the transfection of si-SPRED2. ALP activity (Fig. 4C) was also reduced by down-regulated SPRED2. It was also found that miR-140-3p knockdown attenuated ALP activity which could be reversed by si-SPRED2. Moreover, protein level of COL-I, OPN, Runx2, OCN was decreased by si-SPRED2 compared to NC control in cells treated with 20% PRP (Fig. 4D). Down-regulated miR-140-3p was also proven to enhance the expression of above proteins related to osteogenic differentiation that could be reversed by si-SPRED2. In addition, knockdown of si-SPRED2 suppressed calcium deposition, and miR-140-3p inhibitor accelerated the formation of calcium nodules but it was reversed by si-SPRED2 (Fig. 4E). these finding illustrated that inhibition of miR-140-3p enhanced cell proliferation and osteogenic differentiation of BMSCs by negatively regulating SPRED2 expression.

Knockdown of miR-140-3p enhances autophagy in human BMSCs through negatively regulating SPRED2 expression

Finally, the molecular mechanism of miR-140-3p on autophagy in BMSCs was studied. As shown in Fig. 5A, the expression of SPRED2 mRNA was decreased in cells treated with 20% PRP but up-regulated in cells transfected with miR-140-3p inhibitor, which was reversed by the co-transfection of si-SPRED2. Furthermore, western blotting revealed that the expression of SPRED2 and beclin1 as well as the conversion rate of LC3 I to LC3 II was notably decreased in cells treated with PRP, however, the levels of

them were elevated in cells transfected with miR-140-3p inhibitor but was decreased by the co-transfection of si-SPRED2. These findings indicated that miR-140-3p suppression enhanced autophagy in BMSCs through negatively regulating SPRED2 expression.

Discussion

BMSCs are a type of primary stem cells with good differentiation potential, widely using in tissue engineering for translational research and clinical applications [12]. In recent years, several studies reported the roles of various proteins and miRNAs in the treatment of bone defect [13, 14], however, the mechanism of miRNA-140-3p, SPRED2 and thrombin in BMSCs as well the target relationship still remains unclear. In the present study, we demonstrated that thrombin-activated platelet rich plasma (PRP) enhanced osteogenic differentiation of bone marrow mesenchymal stem cells through miR-140-3p/SPRED2-mediated autophagy.

MiRNAs are reported to be involved in a variety of biological processes, including cell viability, apoptosis and differentiation [15]. MiR-140-3p was reported to regulated osteogenic differentiation of different cells *in vivo* and *in vitro* studie, besides, overexpressed miR-140-3p suppressed the cell viability and differentiation, and enhanced the apoptosis of preosteoblasts cell MC3T3-E1 [16]. It was also found that miR-140-5p restrained osteogenesis mediated by BMP2 in human mesenchymal stem cells [17]. Yin et al found that miR-140-3p was up-regulated in peripheral blood mononuclear of patients with osteoporosis patients, besides, the *in vitro* study showed that miR-140-3p regulated the cell proliferation and differentiation of osteoblasts and osteoclasts by targeting PTEN [18]. Another study found that miR-140-3p was up-regulated in patients with osteoporosis in postmenopausal women and it was considered as a potential biomarker [19]. Though PRP was reported to accelerate osteogenic differentiation in mesenchymal stem cells [20], dental stem cells [21], adipose-derived stem cells [22] and so on, the effect of PRP on miR-140-3p was not investigated and no research illustrating the regulation of miR-140-3p for autophagy in BMSCs. In the present study, we found that activated PRP accelerated osteogenic differentiation, cell proliferation and autophagy by inhibiting miR-140-3p expression. However, there is no research illustrating the regulation of miR-140-3p for autophagy in BMSCs.

Circulating evidences showed that Sprouty-related EVH1 domain-2(SPRED2) was potent inhibitors for cytokines and growth factors [23]. SPRED2 was reported to be involved in various diseases [24]. For example, down-regulated SPRED2 induced cardiac arrhythmias and premature death by suppressing autophagy [25]. SPRED2 interacted with LC3 and enhanced autophagy in tumor cells [26]. In addition, SPRED2 was found to induce erythroid differentiation partly through ERK signaling in chronic myeloid leukemia cells [27]. A previous research showed that miR-221-3p could target SPRED2 and SPRED2 regulated LPS-induced lung inflammation via ERK1/2 pathway [28]. However, no studied reported the regulation relationship between SPRED2 and miR-140-3p in BMSCs. Our study for the first time founder that miR-140-3p negatively regulated SPRED2 expression. Besides, down-regulated miR-140-3p enhanced cell proliferation, osteogenic differentiation and autophagy of BMSCs by negatively regulating SPRED2.

Conclusion

In summary, we for the first time demonstrated that active PRP accelerate osteogenic differentiation of BMSCs by activating autophagy through miR-140-3p/SPRED2 axis. Our findings might bring a new novel for the target therapy for PRP in bone defect repair in future clinic practice.

Abbreviations

BMSCs: bone marrow mesenchymal stem cells; DMEM: Dulbecco's medium Eagle's media; DMSO: Dimethylsulfoxide; FBS: Foetal bovine serum; PBS: Phosphate-buffered saline; qRT-PCR: Reverse Transcription-Quantitative Polymerase Chain Reaction; PRP: platelet rich plasma; SPRED2: Sprouty-related EVH1 domain-2; ATCC: American Type Culture Collection; ALP: Alkaline phosphatase; mRNA: messenger RNA; miR: MicroRNA; 3'UTR: 3'-Untranslated region; WB: Western blotting

Declarations

Ethics approval and consent to participate

The cells were commercially sourced, and the carried work was approved by the Ethics Committee on Henan Provincial People's Hospital.

Consent for publication

Not applicable

Competing interests

The authors declare no conflict of interest.

Data Availability

All data can be obtained from the manuscript or from request to the author.

Funding

None

Authors' contributions

Shuting Jiang wrote the original manuscript and conducted the experiments; Hongyan Liu design the study and reviewed the draft; Weiyan Zhu collected the data and conducted analysis; Hui Yan performed statistical analysis and reviewed the manuscript; Beizhan Yan collected data and reviewed manuscript.

Acknowledgements

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Figures

Figure 1

PRP promotes the proliferation and osteogenic differentiation of human BMSCs by miR-140-3p inhibition or autophagy activation. (A) The transfection efficiency was detected by qRT-PCR. (B) The expression of miR-140-3p was determined by qRT-PCR in BMSCs treated with different concentrations of PRP, BMSCs treated with 20% PRP and transfected with miR-140-3p mimics, BMSCs treated with 20% PRP and transfected with NC mimics, and BMSCs treated with 20% PRP and 3-MA. (C) Cell proliferation was examined by MTT assay. (D) ALP activity was detected. (E) Expression of COL-1, OPN, Runx2 and OCN was measured by qRT-PCR. (F) Calcium deposition was evaluated by Alizarin red staining assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2

PRP activates autophagy and up-regulated SPRED2 expression of human BMSCs through suppressing miR-140-3p expression. (A) Expression of SPRED2 mRNA was detected by qRT-PCR. (B) Western blotting was used to evaluate the protein level of SPRED2, LC3 I, LC3II and Beclin1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3

MiR-140-3p negatively regulated SPRED2 expression. (A) The predicted binding sites between miR-140-3p and SPRED2 are illustrated. (B) Cells were transfected with miR-140-3p mimics, miR-140-3p inhibitor or corresponding NCs, respectively. The targeting relationship between miR-140-3p and SPRED2 was identified by luciferase reporter assay. (C) Expression of SPRED2 was detected by qRT-PCR. (D) Protein expression was determined by western blotting. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 4

Inhibition of miR-140-3p enhances cell proliferation and osteogenic differentiation of BMSCs by negatively regulating SPRED2 expression. (A) Cells were transfected with si-SPRED2 or si-NC. The transfection efficiency was detected by qRT-PCR and western blotting. (B) Cell proliferation was determined by MTT assay in cells treated with 20% PRP, cells treated with 20% PRP and transfected with si-SPRED2 or si-NC, cells transfected with miR-140-3p inhibitor with/without si-SPRED2 and cells transfected with NCs. (C) ALP activity was evaluated. (D) Expression of COL-1, OPN, Runx2 and OCN was

measured by qRT-PCR. (E) Calcium deposition was evaluated by Alizarin red staining assay. *P<0.05, **P<0.01, ***P<0.001.

Figure 5

Knockdown of miR-140-3p enhances autophagy in human BMSCs through negatively regulating SPRED2 expression. (A) Expression of SPRED2 was detected by qRT-PCR. (B) Western blotting was used to evaluate the protein level of SPRED2, LC3 I, LC3II and Beclin1. *P<0.05, **P<0.01, ***P<0.001.