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# Hsa-miR-203 inhibits fracture healing via targeting PBOV1

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**Abstract. – OBJECTIVE: To explore the role of hsa-miR-203 in fracture healing and its underlying mechanism.**

**PATIENTS AND METHODS: Expression levels of hsa-miR-203 and PBOV1 in patients with hand fractures and intra-articular fractures after treatment were detected by quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR). Viability and apoptosis of osteoblast cell line hFOB1.19 after hsa-miR-203 overexpression or knockdown were detected by cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. The target gene of hsa-miR-203 was predicted by bioinformatics and verified by dual-luciferase reporter gene assay. Rescue experiments were conducted to further verify whether hsa-miR-203 could participate in fracture healing via PBOV1.**

**RESULTS: No significant hsa-miR-203 expression was found in patients with hand fractures and intra-articular fractures after treatment for 7 days, which was remarkably upregulated on the 14th day. PBOV1 expression was gradually downregulated as treatment time prolongation. Overexpression of hsa-miR-203 decreased cell viability, but induced apoptosis of hFOB1.19 cells. Bioinformatics predicted that PBOV1 might be the target gene of hsa-miR-203, which was further verified by dual-luciferase reporter gene assay. The effect of hsa-miR-203 on viability and apoptosis of hFOB1.19 cells was reversed after the PBOV1 knockdown.**

**CONCLUSIONS: Hsa-miR-203 inhibits fracture healing by regulating osteoblast viability and apoptosis via targeting PBOV1.**

*Key Words:*
Fracture healing, Hsa-miR-203, PBOV1, MicroRNA.

## Introduction

Fragility fracture is caused by a minor trauma, such as a fall below the center of the body. Fragility fracture has become a major public health problem in the world, which poses a great burden on the society[1,2]. In the United States, there are about 2 million cases of fragility fractures each year, which will increase to 3 million by 2025[3]. Fractures can lead to walking limitations, depression, loss of independence, and chronic pain. Almost all fracture patients need to receive long-term treatment in hospital with a slow recovery process[3-5]. Although there are many strategies to improve the treatment of fragility fractures to accelerate the bone regeneration process, many defects in the treatment plan are still existed[6]. Therefore, it is urgent to study the mechanism of fracture development, so as to find a more effective and practical bone regeneration approach.

MicroRNAs are small-molecule RNAs with approximately 16-25 bases in length, which are widely present in eukaryotes. MicroRNAs exert their biological effects by binding to the 3'-UTR of the corresponding target mRNA at the post-translational level[7,8]. It is reported[9-11] that microRNAs are involved in the regulation of cell cycle, proliferation, differentiation, maturation, and apoptosis. Meanwhile, they also participate in many biological behaviors, such as embryonic development and tumor formation.

Several studies have suggested that microRNAs may exert a vital role in fracture healing. For example, intravenous administration of miR-146a has a remarkable inhibitory effect on the destruction of cartilage and bone, thus improving therapeutic effect on rheumatoid arthritis[12]. Downregulation of miR-9 and miR-181a can promote the cell survival of osteoclasts[13]. Other studies have shown that miR-92a knockdown can significantly promote angiogenesis, thereby accelerating fracture healing in young mice[14]. Our work aims to study the role of hsa-miR-203 in fracture healing and to explore its potential mechanism.

## Patient and Methods

### Sample Collection

75 cases of fragility fracture treated in The Second Hospital of Jilin University from March

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2016 to March 2017 were selected, including 40 cases of hand fracture and 35 of intra-articular fracture. Plasma samples of each patient were collected on the 1st, 7th, 14th, and 21st day, respectively. Plasma samples of healthy controls without treatments for heart failure, kidney failure, joint pain or autoimmune diseases were collected as the control group. This study was approved by our Hospital Ethics Committee and all the subjects signed the informed consent.

### Cell Culture and Transfection

Osteoblast cell line hFOB1.19 was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 1000 U/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in a 5% $CO_2$ incubator at 37°C. For cell transfection, hFOB1.19 were seeded in the 6-well plates for overnight culture. After cell density was up to 50%-60%, corresponding plasmid was transfected according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

### RT-PCR (Real-Time Polymerase Chain Reaction)

We used TRIzol (Invitrogen, Carlsbad, CA, USA) to extract total RNA for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). RNA concentration was determined by a spectrophotometer (Hitachi, Otsu, Shiga, Japan) and those with 1.8-2.1 of A260/A280 were considered with high purity. The expression level of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method. Primers used in the study were as follows: Hsa-miR-203: F: TGCTCTAGAGGCGTCTAAGGCGTCCG, R: CCCAAGCTTCACCTCCCAGCAGCACTTG; U6: F: GCTTCGGCAGCACATATACTAAAAT, R: CGCTTCAGAATTTGCGTGTCAT; PBOV1: F: TGAGTCCCCTCTCGGTAATG, R: GCCCCGAGTTAAGAACATCA; GAPDH: F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

### Cell Counting Kit-8 (CCK-8) Assay

Transfected hFOB1.19 cells were seeded in the 96-well plates with 5000 cells per well. After cell transfection for 1, 2, and 3 days, respectively, 10 μL of CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added in each well. After incubation for 2 h, the OD (optical density) value of each well was measured at the wavelength of 450 nm by a microplate reader (Bio-Rad, Hercules, CA, USA).

### Cell Apoptosis Detection

Transfected hFOB1.19 cells were collected and washed three times with PBS (phosphate buffer saline). Cells were first incubated with 195 μL of Annexin V binding buffer in the dark, followed by incubation with 5 μL of V-FITC and 10 μL of PI for 20 min. Cell apoptosis was detected by flow cytometry (BD Biosciences, Detroit, MI, USA) and analyzed by Flow J software.

### Western Blot

Transfected cells were lysed using a cell lysis buffer, shaken on ice for 30 min, and centrifuged at 4°C, 14,000 ×g for 15 min. Total protein concentration was calculated by BCA (bicinchoninic acid) protein assay kit (Pierce Biotechnology, Rockford, IL, USA). The extracted proteins were separated on a 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and subsequently transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Western blot analysis was performed according to standard procedures.

### Dual-Luciferase Reporter Gene Assay

Wild-type PBOV1 (PBOV1-WT) and mutant-type PBOV1 (PBOV1-MUT) were constructed by Ribobio, Guangzhou, China. The hFOB1.19 cells were co-transfected with hsa-miR-203 mimic or hsa-miR-203 inhibitor with PBOV1-WT or PBOV1-MUT, respectively. Culture medium was replaced 4 h later. Luciferase activity was detected based on the instructions of dual-luciferase reporter gene assay kit (Promega, Madison, WI, USA).

### Statistical Analysis

SPSS (Statistical Product and Service Solutions) 12.0 software (SPSS Inc., Chicago, IL, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Continuous variables were analyzed with the *t*-test. $p < 0.05$ was considered statistically significant.

## Results

### Expressions of hsa-miR-203 and PBOV1 in Fracture Patients

Plasma expressions of hsa-miR-203 and PBOV1 in fracture patients were detected by qRT-PCR. We did not observe remarkable expression change of hsa-miR-203 in hand fracture patients after fixation treatment for 7 days.

However, hsa-miR-203 expression was remarkably upregulated on the 14th day (Figure 1A). A similar change was seen in intra-articular fracture patients (Figure 1B). PBOV1 expression was gradually decreased in hand fracture and intra-articular fracture patients after fixation treatment (Figure 1C and 1D).

### Hsa-miR-203 Inhibited Proliferation and Promoted Apoptosis of hFOB1.19 Cells

Previous studies[15,16] have suggested that microRNAs can function as cell-cell mediators and participate in cellular biological functions such as proliferation and apoptosis. Transfection efficacies of hsa-miR-203 mimic and hsa-miR-203 inhibitor in hFOB1.19 cells were first verified (Figure 2A). We found that overexpressed hsa-miR-203 reduced the viability of hFOB1.19 cells (Figure 2B). Flow cytometry showed that hsa-miR-203 overexpression remarkably induced osteoblast apoptosis (Figure 2C).

### PBOV1 Was the Target Gene of hsa-miR-203

Bioinformatics predicted that 3'UTR of PBOV1 might be the target site of hsa-miR-203. PBOV1-WT and PBOV1-MUT were first constructed (Figure 3A). Transfection of si-PBOV1 remarkably inhibited PBOV1 expression in hFOB1.19 cells (Figure 3B).

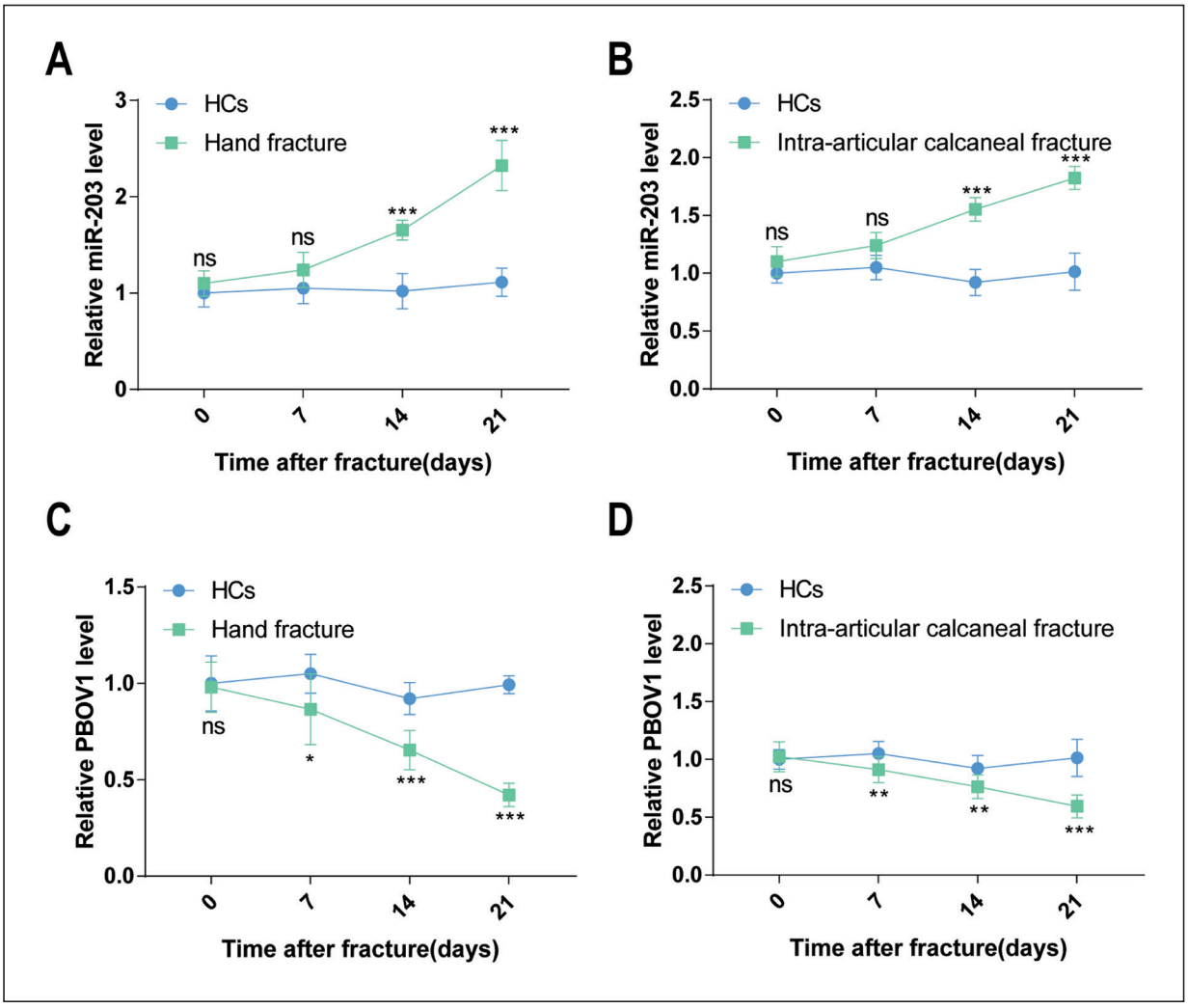


**Figure 1.** Expressions of hsa-miR-203 and PBOV1 in fracture patients. ***A, B,*** Plasma expressions of hsa-miR-203 was remarkably upregulated on the 14th day after fixation treatment in hand fracture patients (***A***) and intra-articular fracture patients (***B***). ***C, D,*** PBOV1 expression was gradually decreased in hand fracture (***C***) and intra-articular fracture patients (***D***) after fixation treatment.

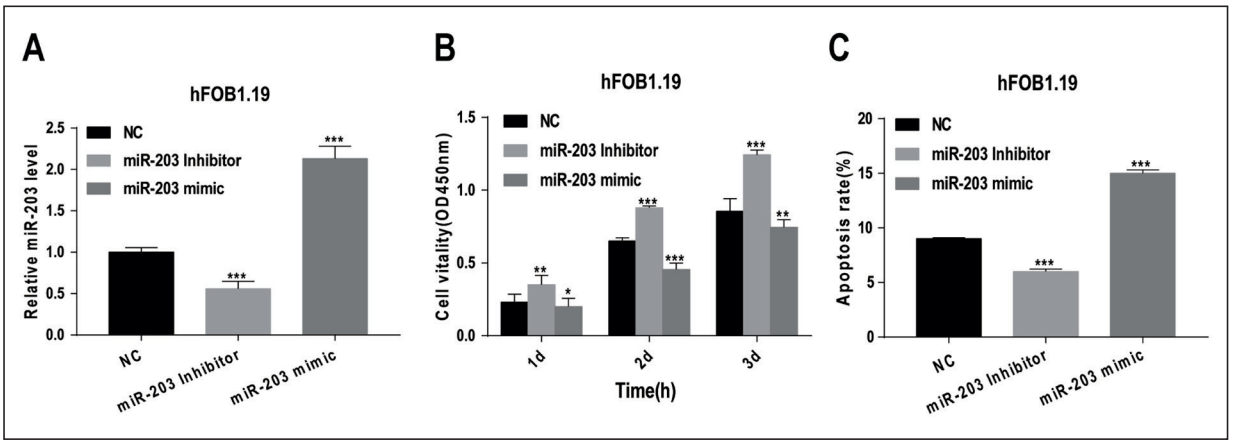


**Figure 2.** Hsa-miR-203 inhibited proliferation and promoted apoptosis of hFOB1.19 cells. ***A,*** Transfection efficacies of hsa-miR-203 mimic and hsa-miR-203 inhibitor in hFOB1.19 cells were verified. ***B,*** Overexpressed hsa-miR-203 reduced viability of hFOB1.19 cells. ***C,*** Flow cytometry showed that hsa-miR-203 overexpression remarkably induced osteoblast apoptosis.

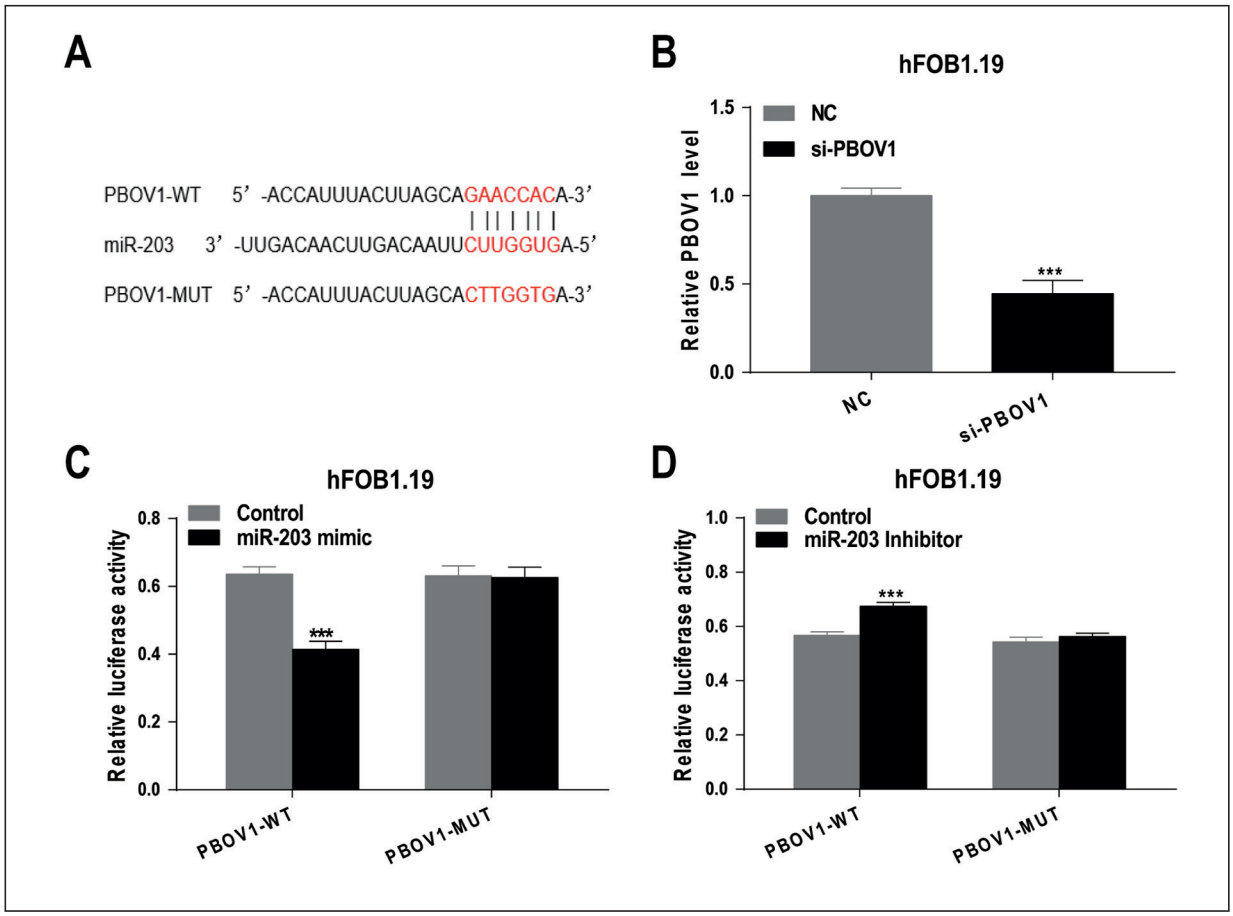


**Figure 3.** PBOV1 was the target gene of hsa-miR-203. ***A,*** PBOV1-WT and PBOV1-MUT were constructed. ***B,*** Transfection of si-PBOV1 remarkably inhibited PBOV1 expression in hFOB1.19 cells. ***C,*** Luciferase activity in hFOB1.19 cells co-transfected with PBOV1-WT and hsa-miR-203 mimic was remarkably decreased compared with that of other groups. ***D,*** Elevated luciferase activity was found in hFOB1.19 cells co-transfected with PBOV1-WT and hsa-miR-203 inhibitor.

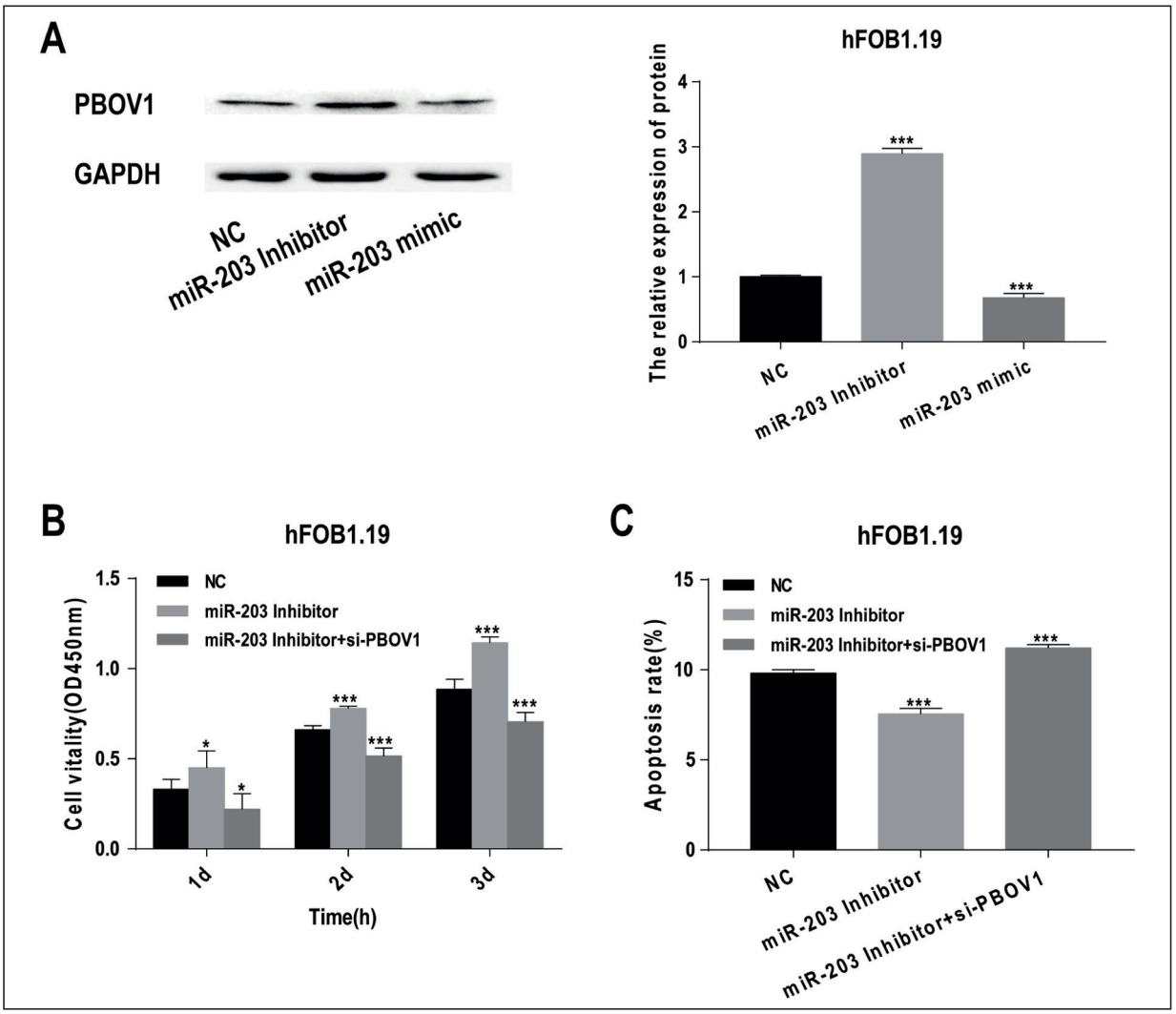


**Figure 4.** Interaction between hsa-miR-203 and PBOV1. ***A,*** Overexpression of hsa-miR-203 resulted in decreased protein level of PBOV1, whereas hsa-miR-203 knockdown obtained the opposite results. ***B,*** PBOV1 expression in hFOB1.19 cells was remarkably reduced after si-PBOV1 transfection. ***B, C,*** The effects of hsa-miR-203 on osteoblast proliferation (***B***) and apoptosis (***C***) were reversed by si-PBOV1 transfection.

Dual-luciferase reporter gene assay indicated that luciferase activity in hFOB1.19 cells co-transfected with PBOV1-WT and hsa-miR-203 mimic was remarkably decreased compared with that of other groups (Figure 3C). On the contrary, elevated luciferase activity was found in hFOB1.19 cells co-transfected with PBOV1-WT and hsa-miR-203 inhibitor (Figure 3D).

### *Interaction Between hsa-miR-203 and PBOV1*

Overexpression of hsa-miR-203 resulted in a decreased protein level of PBOV1, whereas hsa-miR-203 knockdown obtained the opposite results (Figure 4A). Rescue experiments were conducted to study the interaction between hsa-miR-203 and PBOV1 further. First, we found that PBOV1 expression in hFOB1.19 cells was remarkably reduced after si-PBOV1 transfection (Figure 3B). The effects of hsa-miR-203 on osteoblast proliferation and apoptosis were reversed by si-PBOV1 transfection (Figure 4B and 4C).

## Discussion

The rapidly increased incidence of fragility fracture is mainly related to the reduction of physical labor, vitamin D deficiency, and aging of the elderly. Vertebral fracture is the most com-

mon type of fragility fracture, among which, a hip fracture can lead to permanent disability in 30% of patients and loss of independent activity in 80% of patients. Fracture in elderly seriously affects their life quality[4]. Fracture healing requires a variety of growth factors and other types of cytokines to regulate osteoblast proliferation synergistically. Four conditions are considered to be the major factors in successful fracture healing, including suitable mechanical environment, osteoblasts, bone scaffolds, and growth factors for effective induction of osteogenesis[17]. Common fracture healing is a complex process that requires the involvement of a sufficient number of osteoblasts[18].

Hsa-miR-203 is located at 14q32.33, which has been widely studied in recent years[19]. Many researches[20-26] have shown that hsa-miR-203 participates in cell growth, migration, invasion, and radiation resistance. Overexpression of hsa-miR-203 in colorectal cancer cells can significantly induce apoptosis, decrease proliferation and invasion of tumor cells *via* downregulating ZNF217[27]. In lung cancer, hsa-miR-203 acts as an inhibitory gene by inhibiting the expression of the anti-apoptotic protein survivin, and negatively regulating the downstream signaling pathway involved in survivin. Hsa-miR-203 inhibits proliferation, invasion, and metastasis, as well as promotes apoptosis of lung cancer cells[28]. Hsa-miR-203 is downregulated in gastric cancer tissues, which promotes the proliferation of gastric cancer cells[29]. As a tumor suppressor gene, hsa-miR-203 inhibits cell proliferation, migration, and invasion in prostate, kidney, and esophageal squamous cell carcinoma[30-32].

Since hsa-miR-203 is reported to participate in proliferation and apoptosis of tumor cells widely, we speculated whether hsa-miR-203 is involved in regulating osteoblast functions. The present study demonstrated that hsa-miR-203 was upregulated in plasma samples of fracture patients on the 14$^{th}$ day after treatment, indicating its role in fracture healing. Furthermore, we confirmed that hsa-miR-203 inhibited proliferation, but induced apoptosis of hFOB1.19 cells. Bioinformatics predicted that PBOV1 might be the target gene of hsa-miR-203. We detected PBOV1 expression in plasma samples of fracture patients, which was gradually downregulated with the treatment time prolongation. Rescue experiments subsequently demonstrated that hsa-miR-203 regulates osteoblast functions *via* targeting PBOV1.

## Conclusions

We found that hsa-miR-203 inhibits fracture healing by regulating osteoblast viability and apoptosis *via* targeting PBOV1.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

## References


1) Dempster DW. Osteoporosis and the burden of osteoporosis-related fractures. Am J Manag Care 2011; 17 Suppl 6: S164-S169.
2) Kanis JA, Oden A, McCloskey EV, Johansson H, Wahl DA, Cooper C. A systematic review of hip fracture incidence and probability of fracture worldwide. Osteoporos Int 2012; 23: 2239-2256.
3) Hernlund E, Svedbom A, Ivergard M, Compston J, Cooper C, Stenmark J, McCloskey EV, Jonsson B, Kanis JA. Osteoporosis in the european union: medical management, epidemiology and economic burden. A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation Of Pharmaceutical Industry Associations (EFPIA). Arch Osteoporos 2013; 8: 136.
4) Cooper C, Harvey NC. Osteoporosis risk assessment. BMJ 2012; 344: e4191.
5) Ge DW, Wang WW, Chen HT, Yang L, Cao XJ. Functions of microRNAs in osteoporosis. Eur Rev Med Pharmacol Sci 2017; 21: 4784-4789.
6) Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. BMC Med 2011; 9: 66.
7) Huntzinger E, Izaurralde E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet 2011; 12: 99-110.
8) Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature 2010; 466: 835-840.
9) Shiiba M, Shinozuka K, Saito K, Fushimi K, Kasamatsu A, Ogawara K, Uzawa K, Ito H, Takiguchi Y, Tanzawa H. MicroRNA-125b regulates proliferation and radioresistance of oral squamous cell carcinoma. Br J Cancer 2013; 108: 1817-1821.
10) Zhang GJ, Xiao HX, Tian HP, Liu ZL, Xia SS, Zhou T. Upregulation of microRNA-155 promotes the migration and invasion of colorectal cancer cells through the regulation of claudin-1 expression. Int J Mol Med 2013; 31: 1375-1380.
11) Kim JH, Park SG, Song SY, Kim JK, Sung JH. Reactive oxygen species-responsive miR-210 regulates proliferation and migration of adipose-derived stem cells via PTPN2. Cell Death Dis 2013; 4: e588.

12) Pauley KM, Cha S. Mirna-146a in rheumatoid arthritis: a new therapeutic strategy. Immunotherapy-Uk 2011; 3: 829-831.

13) Wang S, Tang C, Zhang Q, Chen W. Reduced mir-9 and miR-181a expression down-regulates Bim concentration and promote osteoclasts survival. Int J Clin Exp Pathol 2014; 7: 2209-2218.

14) Murata K, Ito H, Yoshitomi H, Yamamoto K, Fukuda A, Yoshikawa J, Furu M, Ishikawa M, Shibuya H, Matsuda S. Inhibition of miR-92a enhances fracture healing via promoting angiogenesis in a model of stabilized fracture in young mice. J Bone Miner Res 2014; 29: 316-326.

15) Turchinovich A, Samatov TR, Tonevitsky AG, Burwinkel B. Circulating miRNAs: cell-cell communication function? Front Genet 2013; 4: 119.

16) Turchinovich A, Weiz L, Burwinkel B. Extracellular miRNAs: the mystery of their origin and function. Trends Biochem Sci 2012; 37: 460-465.

17) Giannoudis PV, Einhorn TA, Schmidmaier G, Marsh D. The diamond concept--open questions. Injury 2008; 39 Suppl 2: S5-S8.

18) Shirley D, Marsh D, Jordan G, McQuaid S, Li G. Systemic recruitment of osteoblastic cells in fracture healing. J Orthop Res 2005; 23: 1013-1021.

19) Liang Y, Yang W, Zhu Y, Yuan Y. Prognostic role of microRNA-203 in various carcinomas: evidence from a meta-analysis involving 13 studies. Springerplus 2016; 5: 1538.

20) Viticchie G, Lena AM, Latina A, Formosa A, Gregersen LH, Lund AH, Bernardini S, Mauriello A, Miano R, Spagnoli LG, Knight RA, Candi E, Melino G. Mir-203 controls proliferation, migration and invasive potential of prostate cancer cell lines. Cell Cycle 2011; 10: 1121-1131.

21) Wang C, Wang X, Liang H, Wang T, Yan X, Cao M, Wang N, Zhang S, Zen K, Zhang C, Chen X. Mir-203 inhibits cell proliferation and migration of lung cancer cells by targeting pkcalpha. PLoS One 2013; 8: e73985.

22) Qu JQ, Yi HM, Ye X, Zhu JF, Yi H, Li LN, Xiao T, Yuan L, Li JY, Wang YY, Feng J, He QY, Lu SS, Xiao ZQ. MiRNA-203 reduces nasopharyngeal carcinoma radioresistance by targeting IL8/AKT signaling. Mol Cancer Ther 2015; 14: 2653-2664.

23) Shi Y, Tan YJ, Zeng DZ, Qian F, Yu PW. Mir-203 suppression in gastric carcinoma promotes slug-mediated cancer metastasis. Tumour Biol 2015:

24) Tian L, Li M, Ge J, Guo Y, Sun Y, Liu M, Xiao H. Mir-203 is downregulated in laryngeal squamous cell carcinoma and can suppress proliferation and induce apoptosis of tumours. Tumour Biol 2014; 35: 5953-5963.

25) Wan D, Shen S, Fu S, Preston B, Brandon C, He S, Shen C, Wu J, Wang S, Xie W, Chen B, Liya A, Guo Y, Zheng D, Zhi Q, Peng B. MiR-203 suppresses the proliferation and metastasis of hepatocellular carcinoma by targeting oncogene ADAM9 and oncogenic long non-coding RNA HULC. Anticancer Agents Med Chem 2016; 16: 414-423.

26) Zhang F, Yang Z, Cao M, Xu Y, Li J, Chen X, Gao Z, Xin J, Zhou S, Zhou Z, Yang Y, Sheng W, Zeng Y. MiR-203 suppresses tumor growth and invasion and down-regulates MiR-21 expression through repressing Ran in esophageal cancer. Cancer Lett 2014; 342: 121-129.

27) Li Z, Du L, Dong Z, Yang Y, Zhang X, Wang L, Li J, Zheng G, Qu A, Wang C. MiR-203 suppresses ZNF217 upregulation in colorectal cancer and its oncogenicity. PLoS One 2015; 10: e116170.

28) Jin J, Deng J, Wang F, Xia X, Qiu T, Lu W, Li X, Zhang H, Gu X, Liu Y, Cao W, Shao W. The expression and function of microRNA-203 in lung cancer. Tumour Biol 2013; 34: 349-357.

29) Chiang Y, Song Y, Wang Z, Chen Y, Yue Z, Xu H, Xing C, Liu Z. Aberrant expression of miR-203 and its clinical significance in gastric and colorectal cancers. J Gastrointest Surg 2011; 15: 63-70.

30) Xiang J, Bian C, Wang H, Huang S, Wu D. MiR-203 down-regulates Rap1A and suppresses cell proliferation, adhesion and invasion in prostate cancer. J Exp Clin Cancer Res 2015; 34: 8.

31) Xu M, Gu M, Zhang K, Zhou J, Wang Z, Da J. miR-203 inhibition of renal cancer cell proliferation, migration and invasion by targeting of FGF2. Diagn Pathol 2015; 10: 24.

32) Yuan Y, Zeng ZY, Liu XH, Gong DJ, Tao J, Cheng HZ, Huang SD. MicroRNA-203 inhibits cell proliferation by repressing ΔNp63 expression in human esophageal squamous cell carcinoma. BMC Cancer 2011; 11: 57.