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ORIGINAL ARTICLE

The effects of photobiomodulation therapy on mouse pre-osteoblast cell line MC3T3-E1 proliferation and apoptosis via miR-503/Wnt3a pathway

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Abstract

Photobiomodulation therapy (PBMT) has been demonstrated as regulating osteoblast proliferation. MicroRNAs (miRNAs) are involved in various pathophysiologic processes in osteoblast, but the role of miRNAs in the PBMT-based promotion of osteoblast proliferation remains unclear. This study aimed to investigate the effects of PBMT treatment (3.75 J/cm^2) on mouse pre-osteoblast cell line MC3T3-E1 proliferation and apoptosis via the miR-503/Wnt3a pathway; meanwhile, detect the expressions of miR-503 and Wnt3a after PBMT treatment and the role of miR-503 in regulating Wnt signaling molecules Wnt3a, β-catenin, Runx2, apoptotic proteins caspase-3, and Bcl-2 in vitro. The PBMT parameters were as follows: 808 nm continuous wavelength, 0.401 W output power, 0.042 W/cm^2 power density, 9.6 cm^2 spot size, 36 J energy, 3.75 J/cm^2 energy density, 90 s irradiation for three times per 12 h, 14.5 cm distance of the laser source and the angle of divergence of the laser beam was 7°. In our present study, the target relationship was predicted and verified by bioinformatics analysis and luciferase reporter assays. Gene mRNA and protein expressions were examined by qPCR and western blot analysis. The MTT method was used to evaluate the effect of miR-503 on MC3T3-E1 cells proliferation. And cell apoptosis was examined by flow cytometry. The results showed that PBMT treatment reduced the expression of miR-503 and increased the level of Wnt3a ($p < 0.01$). Bioinformatics analysis and luciferase reporter assays revealed that Wnt3a was a target of miR-503, and Wnt3a was regulated by miR-503. Furthermore, miR-503 was found to functionally inhibit proliferation and promote apoptosis ($p < 0.01$). And during this process, Wnt3a, β-catenin, Runx2, and Bcl-2 expressions were significantly inhibited ($p < 0.01$); however, caspase-3 level was upregulated ($p < 0.01$). These results suggest that miR-503 plays a role in osteoblast proliferation and apoptosis in response to PBMT, which is potentially amenable to therapeutic manipulation for clinical application.

Keywords PBMT · miR-503 · Wnt3a · Signaling pathway · Proliferation

Introduction

PBMT is a form of photon therapy which has been reported to offer numerous benefits in clinical practice, including pain relief [1, 2], anti-inflammation [3], regeneration of severed nerves [4], and wound healing [5, 6]. Until now, lasers have been broadly used in medical fields, including orthopedics and dentistry [7–9]. PBMT has been found to modulate various biological processes in cell culture and animal models [10, 11]. Studies show that PBMT can alter DNA synthesis and gene expression and exert strong effects on cell proliferation [12]. And we previously find that PBMT can promote mouse pre-osteoblast cell line MC3T3-E1 proliferation and demonstrate that lasers of 3.75 J/cm^2 can significantly promote proliferation and affect MC3T3-E1 cell cycle [13]. Meanwhile, the changes in the expression level of microRNAs may be one

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of the consequences of PBMT in cells level [14, 15]. In addition, researches confirm that PBMT plays an important role in soft tissue healing and bone metabolism [16, 17]. PBMT has proven to be a safe and effective therapeutic option in clinical and histologic trials; however, the mechanism of PBMT treatment for cell proliferation and apoptosis remains unclear.

MicroRNAs (miRNAs) are a class of 22-25 nucleotides length non-coding RNAs with functions in post-transcriptional regulation through binding to the 3′-untranslated region (UTR) of the target mRNAs, resulting in mRNA degradation or translation inhibition. Mounting evidence confirmed that miRNAs are key regulators in various biological processes, including cell proliferation [18], apoptosis [19], differentiation [20], and tumor formation [21]. Recent studies suggest that miRNAs may also be associated with bone formation and osteoblast differentiation [22–24]. MiR-503 has been demonstrated as an inhibitor in stretch-induced osteogenic differentiation and bone formation [25]. However, investigations into the role of miR-503 in PBMT pro-proliferation bio-modulation are lacking.

Wnt proteins constitute a family of 19 highly conserved secreted signaling proteins, which can activate either β-catenin dependent canonical signaling pathway or β-catenin independent non-canonical pathways to perform their diverse functions [26]. Several studies have identified that canonical Wnt signaling can promote or inhibit osteoblast differentiation, enhance osteoblast proliferation, maturation, and mineralization, and inhibit osteoblast apoptosis [27, 28]. In addition, Wnt1, Wnt3a, and Wnt10b are found to preferentially activate this canonical Wnt signaling for promoting bone formation [29–31].

However, very little is known about the change of Wnt signaling pathway in osteoblasts which treat with PBMT. In this study, we will predict and verify the target relationship between miR-503 and Wnt3a. Meanwhile, evaluating whether PBMT affects the Wnt signaling pathway via miR-503/Wnt3a, thereby regulating MC3T3-E1 cell proliferation and apoptosis.

Materials and methods

Laser irradiation

A gallium-aluminum-arsenide (Ga-Al-As) diode laser device (near-infrared spectrum) made by Changchun Institute of Optics Fine Mechanics and Physics was used for MC3T3-E1 cells PBMT treatment. The PBMT parameters were as follows: 808 nm continuous wavelength, 0.401 W output power, 0.042 W/cm^2 power density, 9.6 cm^2 spot size, 36 J energy, 3.75 J/cm^2 energy density, 90 s irradiation for three times per 12 h, 14.5 cm distance of the laser source and the angle of divergence of the laser beam was 7° [13]. The beam was perpendicular to the bottom of the dish (3.5-cm-diameter Corning dish) and the excitation beam was placed in the center of the culture dish. The energy calculation formula is as follows: ED (J/cm^2) × S (cm^2) = P (W) × T (s) (ED = energy density, S = spot size, P = power, T = time). The control group was not irradiated. Three biological duplication were used and detected in laser irradiation group and control group respectively.

Cell culture and transfection

Mouse MC3T3-E1 cells were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China), plated at a concentration of 1×10^5 per well into 6-well plates and cultured in DMEM/F12 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; PAA, Pasching, Austria) at 37 °C in a humidified atmosphere with 5% CO2. FuGENE HD (Roche, Switzerland) was used to perform cell transfection according to the manufacturer's instructions. For miR-503 effects on MC3T3-E1 cell detection, miR-503 mimics, miR-503 inhibitor, and shNC were used to transfect the MC3T3-E1 cells. Three biological duplication were used and detected in each group.

Study design

Firstly, MC3T3-E1 cells were divided into two groups: control group (not irradiated) and PBMT group (received laser dose of 3.75 J/cm^2, 90-s irradiation) and seeded in culture plates until the cells reached 80% confluence. The expressions of miR-503 and Wnt3a were detected. Secondly, we verified the target relationship between miR-503 and Wnt3a using luciferase reporter system. Finally, we examined the Wnt pathway key factors and apoptotic proteins expression levels, cell proliferation and apoptosis after the cells transfecting with miR-503 mimics, miR-503 inhibitor, and NC.

RNA extraction and qPCR

After PBMT treatment for 36 h or transfection for 24 h, total RNA was extracted with TRIzol reagent (Invitrogen, USA), according to the manufacturer's instructions. RNA was then converted into complementary (cDNA) by using reverse transcription kit (Takara Biological Company, Japan). For miR-503 expression detection, qRT-PCR was performed using a miRNA qRT-PCR Detection Kit (GeneCopoeia, USA). The U6 gene was used as an internal control. For mRNA expression detection, the SYBR Green I Real-Time PCR kit (Biomics, China) was used according to the manufacturer. GAPDH was used as an internal control. And the primer sequences are listed in Table 1. The reaction conditions were 95 °C for 30 s, followed by 30 cycles of 95 °C for 5 s and

Table 1 Primer sequences of real-time PCR

Symbol	Primer	Primer sequence (5′–3′)
miR-503	RT-primer	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCTGCAGTA
	F-primer	ACACTCCAGCTGGGTAGCAGCGGGAACAGTA TGGTGTCGTGGAGTCG
	R-primer	
U6	RT-primer	AACGCTTCACGAATTTGCGT
	F-primer	CTCGCTTCGGCAGCACA
	R-primer	AACGCTTCACGAATTTGCGT
Wnt3a	F-primer	CTCCTCTCGGATACCTCTTAGTG GCATGATCTCCACGTAGTTCCTG
GAPDH	R-primer	ACCACAGTCCATGCCATCAC TCCACCACCCTGTTGCTGTA
	F-primer	
	R-primer	

miR-503 microRNA 503, *U6* RNU6A, *Wnt3a* wingless type MMTV integration site family member 3A, *GAPDH* glyceraldehyde phosphatedehydrogenase

60 °C for 40 s. The relative expression was determined via the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

After transfection for 24 h, total protein was extracted from cells using RIPA buffer (Boster, China) following the manufacturer's instructions. The protein concentration was examined by a BCA Protein Assay Kit (Sigma, Japan). Wnt3a (1:2000 dilution, Abcam, USA), β-catenin (1:5000 dilution, Abcam, USA), Runx2 (1:2000 dilution, Abcam, USA), caspase-3 (1:1000 dilution, Bioss, China), Bcl-2 (1:1000 dilution, Bioss, China), and GAPDH (1:2000 dilution, Bioss, China) were used to determine protein expression. The protein was separated on SDS-polyacrylamide gel electrophoresis (10% acrylamide) and transferred to PVDF membrane (Bio-Red Laboratories Inc., USA). And an ECL Advanced Western Blotting Detection Kit (Invitrogen, USA) was used for immunoblotting. The bands were showed in a Tanon 5200 chemiluminescence image analysis system (Tanon, China).

Luciferase assay

For dual fluorescein verification, miR-503 mimics (Gene Pharma Company, China) and pmiR-RB-REPORT vectors (pmiR-RB-REPORT-Wnt3a-mut: *ATCATGC*, pmiR-RB-REPORT-Wnt3a-WT: *GCUGCUA* or pmiR-RB-REPORT-si) (RiboBio, China) were transfected into MC3T3-E1 cells. The luciferase activity was detected using a luciferase detection kit (Promega, USA) according to the instructions.

Cell proliferation analysis

The MC3T3-E1 cell proliferation was examined after miR-503 transfection in 96-well plates for 0, 12, 24, 48, or 72 h using an MTT assay according to the manufacturer's instructions (Sigma, Germany). Absorbance (OD) was measured at 450 nm with a microplate spectrophotometer (Bio-Rad, Japan).

Apoptosis detection

The experiments are grouped as follows: miR-503 mimics group, miR-503 inhibitor group, and miR-shNC. Cell apoptosis was detected using a Cell Apoptosis Detection Kit (KeyGEN BioTECH, China) and analyzed by flow cytometry according to the instructions.

Statistical analysis

Each experiment was performed in triplicate and repeated in three independent experiments. Data were the mean ± standard error of the mean calculated by SPSS v 22.0 software. One-way analysis of variance (ANOVA) was used to compare the differences among three groups and statistical analyses between two groups were evaluated based on the Student's *t* test, $p < 0.05$ was considered significant.

Results

PBMT downregulates miR-503 and upregulates Wnt3a expression in MC3T3-E1 cells

The MC3T3-E1 cells were treated with PBMT for 24 h, and the expression of miR-503 and Wnt3a was determined after laser radiation. As shown in Fig. 1a, irradiation for 90 s (fluence 3.75 J/cm^2) significantly downregulated the expression of miR-503 and upregulated Wnt3a level compared to the control group ($p < 0.01$). These results indicated that PBMT at 3.75 J/cm^2 potentially played a role by regulating the expression of miR-503 and Wnt3a in MC3T3-E1 cells.

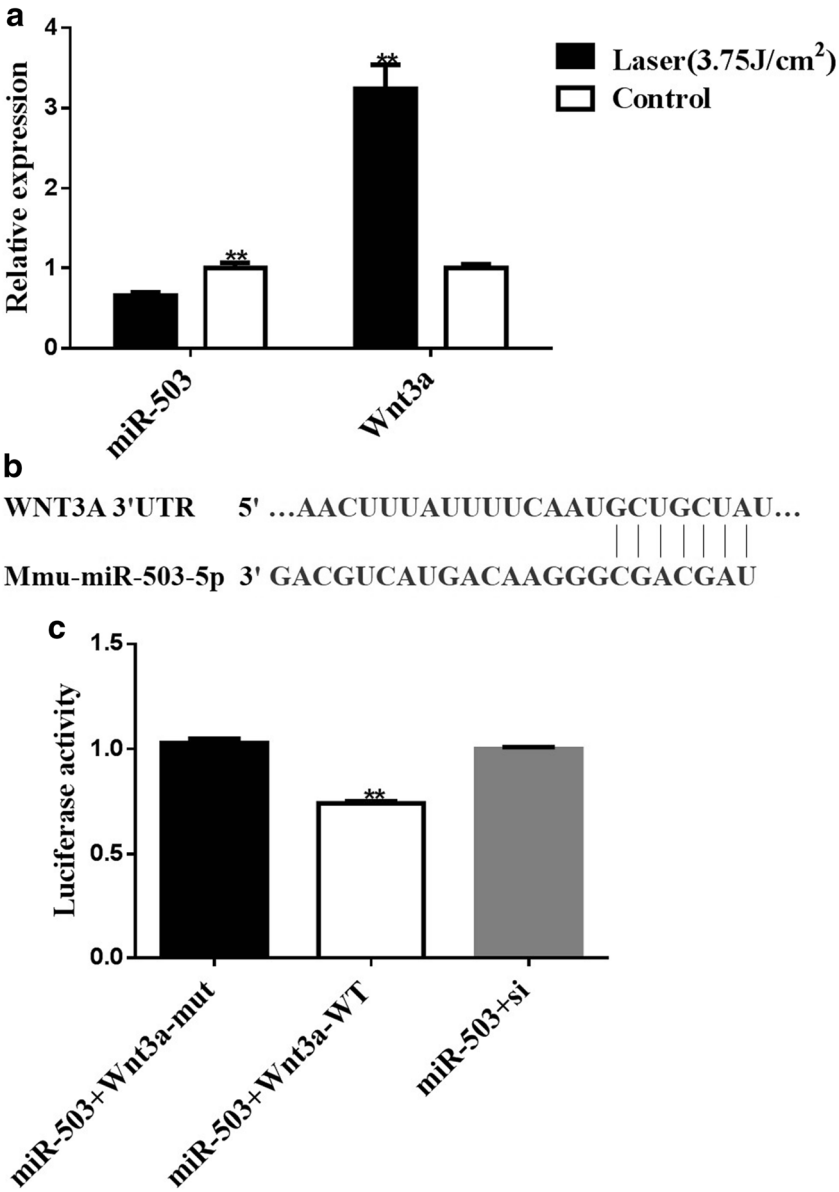

Fig. 1 Effect of PBMT on miR-503 and Wnt3a mRNA expression in MC3T3-E1 cells and target relationship verification between miR-503 and Wnt3a. **a** miR-503 and Wnt3a mRNA expression were detected after PBMT treatment (**$p < 0.01$). **b** MiR-503 and Wnt3a target relationship prediction and luciferase activity detection (**$p < 0.01$)

Wnt3a gene is a target of miR-503

The miR-Base was used to identify miR-503 target genes. Among all the potential genes, Wnt3a was selected for further verification due to its potential roles in cell proliferation and apoptosis and the target sites in 3′UTR of Wnt3a was partially complementary to miR-503 (Fig. 1b). Furthermore, luciferase reporter system was used to test the target site. Compared with the Wnt3a-si/mut group, luciferase activity in cells transfected with Wnt3a-WT vector was significantly decreased ($p < 0.01$) (Fig. 1c). However, no significant difference was found between control and Wnt3a-mut group ($p > 0.05$).

MiR-503 regulates MC3T3-E1 cell proliferation and apoptosis through Wnt signaling pathway

Gene and protein expressions of miR-503, Wnt3a, β-catenin, Runx2, caspase-3, and Bcl-2 were examined by qRT-PCR and western blot analysis after the transfection. The results in Fig. 2 showed that Wnt3a, β-catenin, Runx2, and Bcl-2 expressions were significantly inhibited in miR-503 mimics group ($p < 0.01$); in addition, caspase-3 level was upregulated compared with shNC group ($p < 0.01$). Furthermore, Wnt3a, β-catenin, Runx2, and Bcl-2 expression levels were higher and caspase-3 was lower in miR-503 inhibitor group ($p < 0.01$). The results indicated that

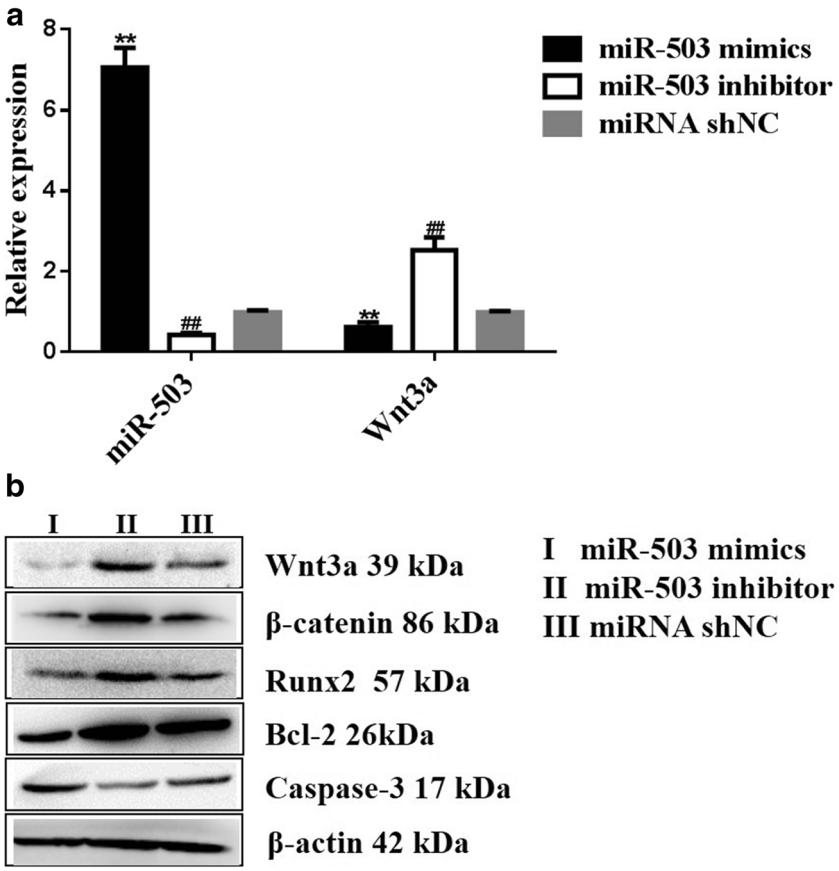

Fig. 2 Relative expression of genes mRNA and proteins in human MC3T3-E1 cells after transfection. MC3T3-E1 cells were transfected with miR-503 mimics, miR-503 inhibitor and miR-shNC. **a** The miR-491 and Wnt3a mRNA expression levels detection results by qPCR (**$p < 0.01$). **b** Western blot analysis for Wnt3a, β-catenin, Runx2, apoptotic protein caspase-3 and Bcl-2

there was a target relationship between miR-503 and Wnt3a. Meanwhile, miR-503 could affect cell proliferation and apoptosis through Wnt signaling pathway.

Cell proliferation and apoptosis

The miR-503 mimics, inhibitor, and miR-shNC were respectively transfected into MC3T3-E1 cells for 24 h. The proliferation and apoptosis of MC3T3-E1 cells was assessed by MTT and flow cytometry. As shown in Fig. 3, the number of MC3T3-E1 cells in the miR-503 mimics group was significantly lower than in the miR-shNC group ($p < 0.01$). The proliferation level of miR-503 inhibitor group was significantly higher than that of miR-shNC group ($p < 0.01$). Meanwhile, miR-503 mimics could significantly promote apoptosis, which was contrary to the role of miR-503 inhibitor ($p < 0.01$).

Discussion

Studies confirm that PBMT promotes the proliferation of osteoblasts by activating the MAPK/ERK pathway [32] and PBMT-mediated BMP/SMAD signaling pathway activation accelerates BMP-induced osteoblast differentiation [33]. Meanwhile, PBMT also activates TAZ and promotes osteoblast differentiation through the Akt/GSK3β signaling pathway [34]. Our previous study demonstrates that 3.75 J/cm^2 PBMT treatment can promote osteoblast proliferation by activating the Hedgehog pathway [34]. However, the mechanisms of cell proliferation induced by PBMT are still poorly understood, in particular, the function of miRNAs in PBMT treatment. Studies have shown that PBMT does have a regulatory effect on the expression of miRNAs [35]. Meanwhile, an increase of Let-7a and miR125a expression and a decrease of miR155, miR21, and miR376b expression after PBMT with the blue laser was found in breast tumor [36]. And miR-193 plays a critical part in MSC proliferation in response to PBMT stimulation [15]. However, no studies on PBMT and miRNAs are found in osteoblasts. This could be the first study that demonstrates the function of miRNA in LLLI treatment in osteoblasts. In this study, miR-503 expression was decreased and Wnt3a expression was increased after PBMT for MC3T3-E1 cells, indicating that miRNAs do participate in the process of PBMT-mediated osteoblasts proliferation.

It is reported that miR-503 plays an important role in tumor occurrence, development, invasion, and metastasis [37–39]. Meanwhile, miR-503 can serve as a therapeutic and diagnostic marker for B-NHL patients due to its high expression

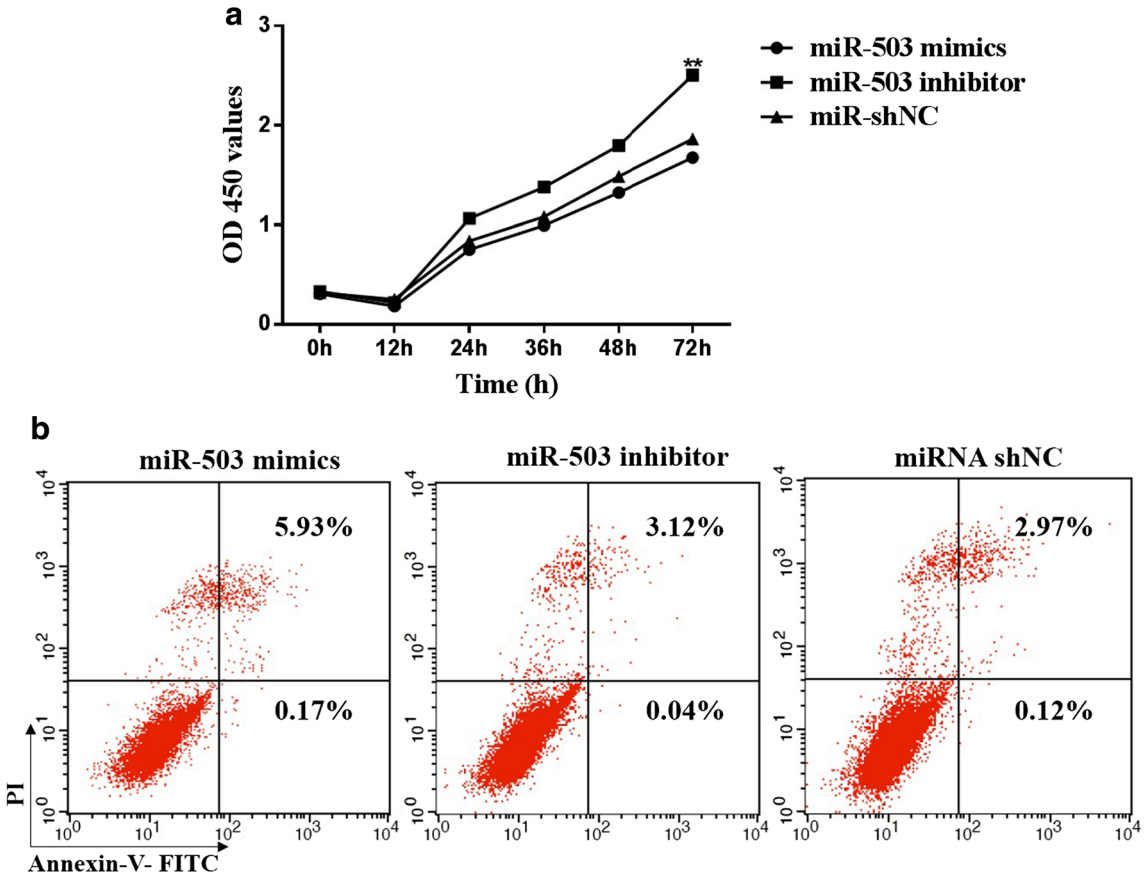

Fig. 3 Cell proliferation and apoptosis detection results. MC3T3-E1 cells were transfected with miR-503 mimics, miR-503 inhibitor, and miR-shNC. **a** MTT assay was used for proliferation detection (**$p < 0.01$). **b** Apoptosis detection results detected by flow cytometer (**$p < 0.01$)

accompanied with reduced expression of KAI1 [40]. In addition, miR-503 also functions as an important epigenetic regulatory factor to promote cardiac fibrosis [41]. In the process of bone absorption and formation, miR-503 inhibits osteoclast differentiation and bone loss by targeting RANK, and on the other hand, miR-503 can significantly increase the activity of the osteoblast marker ALP in the serum and tissues [42]. Furthermore, miR-503 has been demonstrated as an inhibitor in stretch-induced osteogenic differentiation and bone formation [25]. In this study, the overexpression of miR-503 reduced the Wnt3a mRNA and protein levels in MC3T3-E1 cells, and the luciferase reporter assay indicated that Wnt3a was the direct target of miR-503. Moreover, to elucidate functions of miR-503, we detected the proliferation and apoptosis level of MC3T3-E1 cells after transfecting with miR-503, and the results showed that miR-503 inhibited cell proliferation and promoted apoptosis.

The Wnt pathway is closely related to implant osseointegration, and studies have shown that there are many factors that regulate this process, including lithium-based nanoporous coatings on titanium scaffolds and pulsed electromagnetic fields [25, 43–45]. Wnt3a, β-catenin, and Runx2 are important regulatory factors in the Wnt pathway and studies have shown thatWnt3a promotes mesenchymal stem cells, osteoblast precursor cells, and mouse primary osteoblasts cell proliferation and affects cell differentiation [46–49]. In this study, we investigated the expressions of Wnt signaling molecules, including Wnt3a, β-catenin, Runx2, apoptosis protein caspase-3, and Bcl-2, during osteoblast proliferation inhibition induced by miR-503 mimics in MC3T3-E1 cells. This study showed that the overexpression of miR-503 reduced Wnt3a, β-catenin, Runx2, and Bcl-2 expression levels and promoted the expression of caspase-3. Therefore, we believe that miR-503 regulates the proliferation and apoptosis of MC3T3-E1 cells partially through the Wnt pathway. In summary, PBMT was shown to promote MC3T3-E1 cells proliferation and inhibit apoptosis via miRNA-503-Wnt3a and Wnt signaling pathway. The present study provided new insights into the mechanisms of PBMT treatment, and the regulatory role of miRNAs in this process was clarified.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Informed consent Informed consent was obtained from all individual participants included in the study.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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