Original Paper

Plastrum Testudinis Extracts Promote BMSC Proliferation and Osteogenic Differentiation by Regulating Let-7f-5p and the TNFR2/PI3K/AKT Signaling Pathway

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Key Words

Pte • Let-7f-5p • TNFR2/PI3K/AKT signaling pathway • BMSCs • Proliferation • Osteogenic differentiation

Abstract

Background/Aims: Plastrum testudinis extracts (PTE) show osteoprotective effects on glucocorticoid-induced osteoporosis *in vivo* and *in vitro*. However, the underlying molecular mechanism of PTE in promoting osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) is unclear. **Methods:** BMSC proliferation was investigated using the Cell Counting Kit-8 assay. BMSC differentiation and osteogenic mineralization were assayed using alkaline phosphatase and Alizarin red staining, respectively. The mRNA expression levels of *Let-7f-5p*, *Tnfr2*, *Traf2*, *Pi3k*, *Akt*, β -*catenin*, *Gsk3 β* , *Runx2*, and *Ocn* were measured using real time quantitative polymerase chain reaction. Protein levels of TNFR2, TRAF2, p-PI3K, p-AKT, p- β -CATENIN, and p-GSK3 β were analyzed by western blotting. The functional relationship of *Let-7f-5p* and *Tnfr2* was determined by luciferase reporter assays. **Results:** The optimum concentration for PTE was 30 μ g/ml. PTE significantly promoted BMSC osteogenic differentiation and mineralization after 7 and 14 days in culture, respectively. The combination of PTE and osteogenic induction exhibited significant synergy. PTE upregulated *Let-7f-5p*, β -*catenin*, *Runx2*, and *Ocn* mRNA expression, and downregulated *Tnfr2*, *Traf2*, *Pi3k*, *Akt*, and *Gsk3 β* mRNA expression. PTE inhibited TNFR2, TRAF2, and p- β -CATENIN protein expression, and promoted p-PI3K, p-AKT, and p-GSK3 β protein expression. In addition, *Tnfr2* was a functional target of *Let-7f-5p* in 293T cells. **Conclusions:** Our results suggested that PTE may promote BMSC proliferation and osteogenic differentiation via a mechanism associated with the regulation of *Let-7f-5p* and the TNFR2/PI3K/AKT signaling pathway.

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Introduction

A delicate balance between bone formation and resorption, also known as bone homeostasis, is required for maintaining the integrity and physiological functions of the skeletal system. Bone marrow mesenchymal stem cells (BMSCs) are common progenitors of osteoblasts and adipocytes in bone, and impaired osteogenic/adipogenic differentiation can result in imbalanced bone homeostasis [1]. Thus, restoring osteogenic differentiation in BMSCs by targeting known regulatory networks is a promising therapeutic strategy to treat patients with various diseases, such as osteoporosis, arthritis, and Paget's disease [2, 3]. Previously, we showed the protective effect of plastrum testudinis extracts (PTE) in glucocorticoid-induced osteoporosis (GIOP) in rat spine [4, 5]. Moreover, previous studies showed that PTE promoted BMSC proliferation [6, 7]. However, the effect of PTE in promoting BMSC osteogenic differentiation remains largely unexplored, and little is known about the downstream mechanisms of PTE-mediated cell signaling.

Tumor necrosis factor (TNF) is a pleiotropic pro-inflammatory cytokine that is both a powerful stimulator of bone resorption and an inhibitor of bone formation [8]. TNF receptor 2 (TNFR2) is a 75-kDa TNF receptor that exhibits a higher affinity for TNF than does TNFR1 [9]. TNFR2 is preferentially activated by membrane-integrated TNF, which leads to the recruitment of TNF receptor-associated factor 2 (TRAF2) to the intracellular TRAF-binding motif. Stimulation of phosphatidylinositol 3-kinase (PI3K)/AKT signaling occurs after TRAF2 recruitment, which then induces I κ B kinase (IKK) activation, subsequent I κ B α phosphorylation, and nuclear factor kappa B (NF- κ B) activation [10]. Several lines of evidence have suggested that the TNFR2 is a strong candidate gene for involvement in peak bone mineral density (BMD) due to its important role in bone turnover and regulation of the effect of TNF on osteoclastogenesis [11]. Additionally, the TNFR2 gene location has been identified as the potential quantitative trait loci for BMD variation in the hip [12]. Significant associations between the TNFR2 gene and BMD variations have been reported in several populations [13-17]. Moreover, *in vitro* and *in vivo* studies have suggested that TNFR2 expression is associated with mesenchymal stem cell (MSC) proliferation [18]. Thus, we speculate that TNFR2-mediated TNF signaling pathway plays a pivotal role in regulating BMSC proliferation and osteogenic differentiation.

Micro-RNAs (miRNAs) play important roles as posttranscriptional gene regulators in numerous biological processes including cell development, stem cell regulation, and human diseases. miRNAs are mostly transcribed from intragenic or intergenic regions and undergo further processing by Drosha and other downstream ribonucleases before being converted into mature 18- to 25-nt miRNA transcripts that are capable of gene silencing [19]. Specific miRNAs or miRNA expression profiles are closely involved in controlling the key steps in osteogenic differentiation in BMSCs [20, 21]. Importantly, recent studies in human MSCs (hMSCs) demonstrated that specific *Let-7f* miRNAs are upregulated during osteogenic differentiation [22, 23]. Moreover, members of the *Let-7f* family are thought to function as prodifferentiation factors with "antistemness" properties [24]. For example, mature *Let-7f* miRNAs are undetectable in embryonic stem cells but show robust accumulation after the onset of differentiation in these cells [25]. However, few studies have focused on the roles of *Let-7f* in the regulatory networks that control BMSC proliferation and osteogenic differentiation in response to PTE intervention.

In the present study, we showed that PTE acts as a promoter of BMSC proliferation and osteogenic differentiation through positive regulation of *Let-7f-5p* expression and negative modulation of the TNFR2/PI3K/AKT pathway activity. Furthermore, for the first time, we revealed that the underlying regulatory network involved the *Let-7f-5p*, as a key factor targeting the translation of *Tnfr2*.

Materials and Methods

BMSC isolation and in vitro culture

BMSCs were obtained from three randomly selected 6-week-old Sprague-Dawley female rats. After euthanasia by sodium pentobarbital and cervical dislocation, bone marrow was flushed out of the femur and tibia with Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco) and 1% (v/v) penicillin and streptomycin (Gibco) under standard sterile conditions. The mixture of bone marrow and medium was gently added to the a 50% Percoll solution (Sigma-Aldrich St. Louis, MO, USA) and centrifuged at 3,000 rpm for 30 min. The cell suspension was obtained between the layer of Percoll and the supernatant liquid layer. Cells were plated and then incubated in a humidified atmosphere of 5% CO₂ at 37°C. The cells were passaged every 3 to 4 days using 0.25% (w/v) trypsin-EDTA solution (Gibco), and used for experiments at the third or fourth passage.

To induce osteoblastic differentiation, the BMSCs were cultured in an osteogenic induction (OI) medium (growth culture medium supplemented with 10⁻⁸ M dexamethasone) (Sigma-Aldrich), 50 µg/ml ascorbic acid (Sigma-Aldrich), and 5 mM β-glycerol phosphate (Sigma-Aldrich) at an initial density of 1 × 10⁴ cells/cm². The medium was changed every 4 days during osteogenesis.

Preparation of PTE

PTE was obtained according to a method established previously [4-7]. In brief, plastrum testudinis (PT) was purchased from the First Affiliated Hospital of Guangzhou University of Chinese Medicine (Guangzhou, China) and extracted stepwise with ethyl acetate solvent and water; the solvents were recovered to obtain PTE in ethyl acetate, which was dissolved in dimethyl sulphoxide. Gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) were performed for determining steroids, fatty acids, and their ester components. This extract contained nine chemical compounds including palmitic acid methyl ester (PubChem CID: 8181), ethyl palmitate (PubChem CID: 12366), methyl stearate (PubChem CID: 8201), stearic acid ethyl ester (PubChem CID: 8122), palmitic acid (PubChem CID: 985), stearic acid (PubChem CID: 5281), cholesterol (PubChem CID: 5997), cholesterol myristate (PubChem CID: 99486), (+)4-cholesten-3-one (PubChem CID: 91477). The concentrations of PTE used in the study were 0 (as control group), 0.03, 0.3, 3, 30, and 300 µg/mL.

Cell proliferation analyses using the Cell Counting Kit (CCK)8 assay

The proliferation of BMSCs with various concentrations of PTE was measured using the CCK-8 assay (Dojindo Molecular Technologies, Kumamoto, Japan). The BMSCs were cultured in 96-well plates at 1 × 10⁵ cells/mL with growth culture medium. After 2 h of cell adhesion, the cells were incubated in media containing various concentrations of PTE for 1, 3, 5, and 7 days. Next, the BMSCs were seeded in 6-well plates at 1 × 10⁴ cells per well (2 wells). After 2 h of cell adhesion, the cells were incubated in media containing various concentrations of PTE for 14 days. The cells were digested with 0.25% trypsin. Cells in each well were aspirated with 1 mL of culture medium, centrifuged at 800 rpm for 5 min, resuspended in 100 µL of culture medium, and plated in 96-well plates. On the following day, the CCK8 assay was used to detect cell viability. The remaining wells were cultured for 21 days. Cell viability was measured using the CCK8 assay as described by the manufacturer. The culture fluid was aspirated 4 h before the end of cell processing. The CCK-8 assay was performed by incubating in 10% CCK-8 solution at 37°C in a 5% CO₂ incubator for 4 h. For CCK-8 measurement, 100 µL of the incubated cell suspension was transferred to a 96-well plate. The absorbance of the culture medium was measured at 450 nm using a microplate reader. The optimal concentration of PTE was selected for inducing osteogenic differentiation according to the results of the CCK8 assay. The assay groups were treated as follows: (1) the control group received FBS medium, (2) the PTE group received FBS medium with PTE, (3) the OI group received OI medium, and (4) the PTE+OI group received OI medium with PTE.

Alkaline phosphatase (ALP) activity assays

For ALP staining, BMSCs were cultured in 24-well plates at a cell density of 1 × 10⁴ and analyzed on day 21 using an ALP staining kit (Beyotime, Shanghai, China). Cells that were cultured in OI medium were rinsed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde. Briefly, after formalin fixation for 10 min, 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) working

solution was added to each well and incubated in the dark at room temperature for 30 min. The cells were rinsed twice with distilled water, counterstained with Neutral red (Beyotime) for 5 min, and photographed under a microscope.

ALP activity was determined using an ALP assay kit in 6-well plates at a cell density of 1×10^4 . The cells were rinsed three times with cold PBS and lysed in radioimmunoprecipitation assay buffer (RIPA; Sigma-Aldrich) on ice. The total protein concentration was determined using a BCA protein assay kit (Beyotime). The optical density (OD) of the ALP protein was measured at 520nm according to the manufacturer's instruction. ALP activity was calculated using the ALP level normalized to the total protein.

Alizarin red staining

BMSCs were seeded at 1×10^4 cells per well onto 24-well plates, and the medium was changed after 24 h of incubation. Cells in the various groups were fixed with 4% paraformaldehyde for 15 min at 37°C and rinsed three times with distilled water. Then, the fixed cells were stained for 15 min in Alizarin red solution (1.0 g Alizarin powder was dissolved into 50 mL distilled water and adjusted to pH 4.2). Finally, the cells were washed in distilled water to remove excess stain and observed using a microscope. Quantitation was achieved by dissolving the mineralized nodules with hexadecylpyridinium chloride, and the absorbance (OD value) of the liquid was measured at 578 nm.

Real time quantitative polymerase chain reaction (RT-qPCR) analyses

BMSCs were harvested, and total RNA was extracted using the MiniBEST Universal RNA Extraction Kit (Cat. No. 9767; Takara Bio Inc., Japan) according to the manufacturer's protocol. Reverse transcription was performed using 60 µL of reaction solution (up to 500 ng total RNA was contained in 10 µL of reaction solution) with the PrimeScript RT Master Mix kit (Perfect Real Time, Cat No. RR036A; Takara). Real-time analyses were performed using the SYBR Premix Ex Taq™ II kit (TilRNaseH Plus, Cat # RR820A; TaKaRa) on a iQ5 PCR cycler (Bio-Rad, Hercules, CA, USA). A total of 2 µL cDNA was amplified using the primers listed in Table 1. The genes assayed comprised *Let-7f-5p*, *Tnfr2*, *Traf2*, *Pi3k*, *Akt*, *β-catenin*, *Gsk3β*, *Runx2*, and *Ocn*. The RT-qPCR parameters were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. *β-actin* was used as an internal control, and all experiments were performed in triplicate. Relative expression levels of each gene were quantified by using the $2^{-\Delta\Delta Ct}$ method.

Western blot analyses

BMSCs were harvested and lysed using RIPA buffer (Beyotime). Protein levels in the supernatant were determined using the BCA Protein Assay Kit (Tiangen Biotech Co., Ltd., Beijing, China). Equal amounts of proteins (20 µg) were separated on 6-18% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk for 1 h and washed three times with TBST. The membranes were incubated with a primary antibody against TNFR2 (#3727; 1:1, 000; CST, Framingham, MA USA), TRAF2 (#4712; 1:1, 000; CST), p-PI3K (ab182651; 1:1, 000; Abcam, Cambridge, UK), p-AKT (#4060; 1:2, 000; CST), p-β-CATENIN (#9561; 1:1, 000; CST), p-GSK3β (#5558; 1:1, 000; CST), and GAPDH (RC-5G5; 1:10, 000; KangChen Biotech, Shanghai, China), overnight at 4°C. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein expression signals were visualized by enhanced chemiluminescence (ECL; Beyotime). And band intensities were quantified using Image J software (National Institutes of Health, Bethesda, MD, USA).

Plasmid construction

Let-7f-5p mimics and negative control (NC) mimics were synthesized by Biomix Biotech Co., Ltd. (Nantong, China). To generate the luciferase reporter vector, a 220-bp fragment of the *Tnfrsf1b* (*Tnfr2*)

Table 1. Primers used for quantitative RT-qPCR

ID	Sequence (5'-3')
Let-7f-5p	TGAGGTAGTAGATTGTATAGTT
Let-7f-5p-RT	CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGAACTATA
Let-7f-5p-F	ACACTCCAGCTGGGTGAGGTAGTAGATTGTAT
Tnfr2-F	TCAGCCAGAGCCACAAGA
Tnfr2-R	GAAGCAGTTCCGCGTCCTA
Traf2-F	TGAAAGGTCCCAATGATGC
Traf2-R	GGAGGACGAAGTTACATCAGG
Pi3k-F	ACGGCAATGTGGAGCAGA
Pi3k-R	GTCGTAGCCAATCAGGGAG
Akt-F	CTTTATTGGCTACAAGGAACGG
Akt-R	CAGTCTGAATGGCGGTGGT
β-catenin-F	TGCAGCGACTAAGCAGGA
β-catenin-R	TCACCAGCAGCAAGGACA
Gsk3β-F	GTCCGATTGCGGTATTCT
Gsk3β-R	ACAGGAGTGTCTGCTTGG
Runx2-F	GACTGTGGTTACCGTCATGGC
Runx2-R	ACTTGGTTTTTCATAACAGCGGA
Ocn-F	CTGACCTCACAGATCCCAAGC
Ocn-R	TGGTCTGATAGCTCGTCACAAG
β-actin-F	GGAGATTACTGCCCTGGCTCCTA
β-actin-R	GACTCATCGTACTCTGCTTGCTG

mRNA 3' untranslated region (UTR) containing the predicted *Let-7f-5p* binding site (position 794-801) (*Tnfr2* mRNA 3' UTR-WT) and its mutant sequence (*Tnfr2* mRNA 3' UTR-MUT) were synthesized by Genechem and cloned into psoCHECK2 vectors (Promega Biotech Co., Ltd., Beijing, China). The sequences of all constructs were confirmed by DNA sequencing. The primers used are listed in Table 2.

Transfection

For transient transfections, a 20 nM final concentration of *Let-7f-5p* mimics, NC mimics, *Tnfr2* 3' UTR-WT, and *Tnfr2* 3' UTR-MUT in Opti-MEM (Invitrogen, Carlsbad, CA, USA) was combined with Lipofectamine 2000 (Invitrogen) in serum-free medium and incubated at room temperature for 20 min. The mixture was then added to 293T cells in 24- or 6-well plates at 80% fusion. The medium was replaced by proliferation or differentiation medium 6 h later. For long-term detection under differentiation culture conditions, the cells were transfected every 3 days.

Luciferase reporter assays

The 293T cells were transfected with either the *Let-7f-5p* mimics or NC mimics in conjunction with the luciferase reporter constructs. At 48 h post-transfection, the cells were lysed and subjected to luciferase assays using a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The assay was performed in triplicate.

Statistical analysis

The data were analyzed with SPSS software (ver. 19.0; SPSS Inc., Chicago, IL, USA). For quantitative analysis, all results are expressed as mean $\pm$ standard deviations (SD). Statistical significance was evaluated by one-way analysis of variance (ANOVA) with the Student-Newman-Keuls (SNK) test applied for post hoc analysis. Two-sided *P*-values < 0.05 were considered to indicate statistical significant.

Results

PTE promoted BMSC proliferation.

BMSC proliferation was analyzed on days 1, 3, 5, 7, 14, and 21 days (Fig. 1). The relative cell number (OD value) at each concentration of PTE increased gradually in a time-dependent manner until day 7. Greater numbers of BMSCs were observed with treatment by PTE at concentration of 0.03, 0.3, 3, 30, and 300 $\mu\text{g/mL}$ compared with control-treated BMSCs (0 $\mu\text{g/mL}$); however, the differences showed no statistical significance. After 7 days, a dose-dependent decrease in the quantity of BMSCs was observed at each concentration of PTE. BMSC proliferation was lowest in the absence of PTE (0 $\mu\text{g/mL}$) and highest in the presence of 300 $\mu\text{g/mL}$ PTE ($P < 0.05$); however, no significant difference was observed compared with that at the PTE concentration of 30 $\mu\text{g/mL}$. Furthermore, BMSC proliferation showed a steady and slight increase on day 14 when treated with PTE at a concentration of 30 $\mu\text{g/mL}$.

PTE promoted BMSC osteogenic differentiation

We compared the early differentiation of BMSCs after treatment with PTE, OI medium, and the combination of PTE and OI medium by performing ALP staining on days 3, 7, 14, and 21 (Fig. 2A). BMSCs cultured for 7 days in OI group and PTE+OI group showed stronger ALP staining intensity than those in the control and PTE groups. Moreover, after 14 and 21 days in culture, the PTE, OI, and PTE+OI groups showed significantly stronger ALP staining intensity compared with the control group. ALP staining in the PTE+OI group showed the strongest intensity at each time point. Quantitative ALP assays further confirmed the results of ALP staining (Fig. 2B).

We compared the osteogenic mineralization ability of the BMSCs after treatment with PTE, OI medium, and the combination of PTE and OI medium by performing Alizarin red

Table 2. Primers luciferase reporter constructs

ID	Sequence (5'-3')
Tnfrsf1b-MUT-F	GCCACCTGTCTCGTCAATGGCTTGCCTGGAGCACTCTG
Tnfrsf1b-MUT-R	CAGAGTGCTCCAGGCAAGCCATTGACGAGACAGGTGGC
Tnfrsf1b-XhoI-F	CCGCTCGAGTTTATCCTCTGCCTTTGACTTTTACTCC
Tnfrsf1b-NotI-R	ATTGCGGCCGCATTAGAAAATAAGCATGAATCCTTTGGC

Fig. 1. Effect of PTE on BMSC proliferation was analyzed on day 1, 3, 5, 7, 14, and 21. The experiment was repeated for 3 times, and the data were expressed as mean $\pm$ SD * p <0.05 versus the control group. The OD 450 values were measured at the specific time points to draw curves.

Fig. 2. BMSC osteogenic differentiation assay. A. the ALP staining assay of BMSC treated with PTE, OI medium, and the combination of PTE and OI medium for 3, 7, 14, 21 days. Scale bar, 100 μ m. B. Quantitative assay of ALP activity at day 3, 7, 14, and 21. The experiment was repeated for 3 times, and the data were expressed as mean $\pm$ SD * p <0.05 versus the control group, # p <0.05 versus the PTE group, Δ p <0.05 versus the OI group.

Fig. 3. BMSC osteogenic mineralization assay. A. Alizarin red staining of BMSCs treated with PTE, OI medium, and the combination of PTE and OI medium for 3, 7, 14, 21 days. Scale bar, 100 μ m. B. Quantitative assay of Alizarin red staining. The experiment was repeated for 3 times, and the data were expressed as mean $\pm$ SD * p <0.05 versus the control group, # p <0.05 versus the PTE group, Δ p <0.05 versus the OI group.

staining on days 3, 7, 14, and 21 (Fig. 3A). BMSCs cultured for 7, 14, and 21 days formed mineralized nodules in the PTE, OI, and PTE+OI group; the amount of matrix mineralization was highest in the PTE+OI group at each time point. Quantitative measurement of Alizarin red staining further confirmed the results (Fig. 3B).

PTE upregulated Let-7f-5p, β -catenin, Runx2, and Ocn mRNA expression, and downregulated Tnfr2, Traf2, Pi3k, Akt, and Gsk3 β mRNA expression during BMSC osteogenic differentiation

We investigated the mRNA expression levels of *Let-7f-5p*, four target genes of the TNFR2/PI3K/AKT signaling pathway (*Tnfr2*, *Traf2*, *Pi3k*, and *Akt*), β -catenin, *Gsk3 β* , *Runx2*, and *Ocn* after 21 days in culture during osteogenic differentiation (Fig. 4). The PTE, OI, and PTE+OI groups showed significant upregulation of *Let-7f-5p*, β -catenin, *Runx2*, and *Ocn* mRNA expression, but significant downregulation of *Tnfr2*, *Traf2*, *Pi3k*, *Akt*, and *Gsk3 β*

mRNA expression. No differences were detected in the mRNA expressions of *Tnfr2*, *Traf2*, *Akt*, β -catenin, and *Gsk3 β* between the PTE and OI groups. However, the OI group showed significantly lower levels of *Let-7f-5p* and *Pi3k* mRNA compared with the PTE group. Furthermore, the PTE+OI group showed the highest levels of *Let-7f-5p* and β -catenin mRNA, and the lowest levels of *Tnfr2* and *Traf2* mRNA among the experimental groups.

PTE inhibited TNFR2, TRAF2, and p- β -CATENIN protein expression, and promoted p-PI3K, p-AKT, and p-GSK3 β protein expression during BMSC osteogenic differentiation

Western blot assays were performed to investigate the protein expression of TNFR2, TRAF2, p-PI3K, p-AKT, p- β -CATENIN, and p-GSK3 β after 21 days in culture during OI (Fig. 5). Compared with the control group, the PTE, OI, and PTE+OI groups exhibited significantly lower protein expression levels of TNFR2, TRAF2, and p- β -CATENIN, but significantly higher levels of p-PI3K and p-GSK3 β . Additionally, no differences in TNFR2, p-PI3K, or p- β -CATENIN protein expression levels were detected between the PTE and OI groups. Nevertheless, the OI group showed significantly higher protein expression levels of TRAF2, but significantly lower levels of p-AKT and p-GSK3 β compared with the PTE group. Moreover, the PTE+OI group exhibited the lowest protein expression levels of TNFR2, TRAF2, and p- β -CATENIN, but the highest levels of p-PI3K, p-AKT, and p-GSK3 β among the experimental groups.

Let-7f-5p negatively regulated TNFR2 in 293T cells

miRNAs modulate cell proliferation and differentiation by downregulating the expression of downstream targets. Mechanistically, miRNAs bind to the 3' UTR of mRNAs, which leads

Fig. 4. The relative mRNA expression levels of *Let-7f-5p*, four target genes of the TNFR2/PI3K/AKT signaling pathway (*Tnfr2*, *Traf2*, *Pi3k*, and *Akt*), β -catenin, *Gsk3 β* , *Runx2*, and *Ocn* were showed. The experiment was repeated for 3 times, and the data were expressed as mean $\pm$ SD * p <0.05 versus the control group, # p <0.05 versus the PTE group, Δp <0.05 versus the OI group.

Fig. 5. Protein expression levels of TNFR2, TRAF2, p-PI3K, p-AKT, p- β -CATENIN, and p-GSK3 β were analyzed by Western blot. The experiment was repeated for 3 times, and the data were expressed as mean $\pm$ SD * p <0.05 versus the control group, # p <0.05 versus the PTE group, Δp <0.05 versus the OI group.

to consequential mRNA degradation or inhibition of translation. Using the online program TargetScan (data not shown), we identified several potential targets of *Let-7f-5p* including *Tnfr2*, an established transcription factor and main target of *Let-7f-5p*. We targeted the *Let-7f-5p* binding sites at 794–801 of the *Tnfr2* 3' UTR (Fig. 6A) and constructed plasmids containing wild type and mutant *Tnfr2* to transfect 293T cells. Luciferase reporter assays were performed after 48 h transfection. The luciferase reporter assays showed significantly lower luciferase activity in the *Let-7f-5p* and *Tnfr2* cotransfected cells (Fig. 6B), indicating that *Let-7f-5p* bound to the 3' UTR of *Tnfr2*. The complementary binding of the miRNA to the 3' UTR mRNA resulted in degradation of the target gene.

Discussion

This study investigated the mechanism involved in PTE regulation of BMSC proliferation and osteogenic differentiation. PTE stimulated BMSC proliferation in a time- and dose-

Fig. 6. *Let-7f-5p* negatively regulates *Tnfr2* by binding to the *Tnfr2* 3' UTR. A. Schematic of putative *Let-7f-5p* binding sites and mutated binding sites in *Tnfr2* 3' UTR. Putative *Let-7f-5p* binding site and mutated binding sites was indicated with red character. B. Luciferase reporter assay of 293T cells. Column, means; bar, SD. *** $P < 0.01$ vs negative control (NC) mimics.

Fig. 7. The flow chart on the action mechanism of PTE. Differential expression levels of miRNAs cluster (heat map) analyses and volcano plot of vertebral samples of glucocorticoid-induced osteoporosis (GIOP) by performing miRNA-seq analysis and qRT-PCR validation and *Let-7f-5p* was selected. PTE may promote BMSC proliferation and osteogenic differentiation, and the mechanism may be associated with the regulation of *Let-7f-5p* and the *TNFR2*/PI3K/AKT signaling pathway. "↑" represents promoting effect and "↓" represents inhibiting effect.

dependent manner, which peaked at day 7. This result was consistent with that described in a previous study, which showed that PTE treatment for 4 days induced cell proliferation [26]. Moreover, PTE treatment at a concentration of 30 $\mu\text{g/mL}$ slightly and consistently promoted BMSC proliferation from day 7 to 14. This result indicated that the PTE concentration of 30 $\mu\text{g/mL}$ was the optimum concentration, in accordance with a previous study indicating day 14 as the optimal treatment time [6]. However, to the best of our knowledge, no previous studies have reported the role of PTE in regulating BMSC osteogenic differentiation.

ALP and Alizarin red staining showed that treatment with PTE, OI, and PTE+OI significantly promoted BMSC osteogenic differentiation, suggesting synergistic effects of ALP and OI. Moreover, the effect of PTE in promoting BMSC osteogenic differentiation was further verified by the presence of increased mRNA expressions of *β -catenin*, *Gsk3 β* , *Runx2*, and *Ocn*, and increased protein expression of p- β -CATENIN and p-GSK3 β , which are important in the Wnt/ β -catenin signaling pathway [27]. Therefore, PTE may be a promising therapeutic agent against a series of bone metabolic diseases associated with impaired BMSC osteogenic differentiation. This *in vitro* study may shed light on the effect of PTE in rescuing glucocorticoid-induced spinal osteoporosis in rats [4, 5].

Although previous studies suggested the importance of TNFR2 in regulating osteoclastogenesis, peak BMD, and MSC proliferation, the potential mechanism underlying the TNFR2-mediated TNF signaling pathway in BMSC proliferation and osteogenic differentiation requires further elucidation. TRAF2 recruitment is a key step in the TNFR2-mediated TNF- α signal transduction pathway. A dominant-negative mutant of TRAF2 blocks TNFR2-mediated NF- κ B activation, whereas overexpression of TRAF2 causes activation of NF- κ B, suggesting that TRAF2 plays a pivotal role in TNFR2-mediated NF- κ B activation [26]. In addition, PI3K/AKT signaling acts as a central regulator in bone tissue metabolism and proliferation, and osteogenic differentiation of MSCs [28-31]. The present study revealed for the first time that PTE downregulates *Tnfr2*, *Traf2*, *Pi3k*, and *Akt* mRNA expression, and promotes p-PI3K, p-AKT, and p-GSK3 β protein expression during BMSC osteogenic differentiation, demonstrating that the ability of PTE to stimulate osteogenic differentiation in BMSCs is dependent on the TNFR2/PI3K/AKT signaling pathway. Recently, TNFR2 was identified as a key drug target for cancer immunotherapy [32] and various autoimmune diseases [18], suggesting that the TNFR2-mediated TNF signaling pathway may be involved in regulating the immune system and inflammation. Interestingly, along with PTE-induced BMSC osteogenic differentiation, proinflammatory signaling cascades are suppressed by PTE, suggesting that anti-inflammatory therapies may be applicable for treating bone mass loss, which is associated with impaired BMSC osteogenic differentiation [33]. Therefore, PTE has the potential to be an effective anti-inflammatory drug.

To further elucidate the intracellular molecular mechanism by which PTE promotes BMSC proliferation and osteogenic differentiation, we focused on miRNAs, which play important roles in the functionality of BMSCs [20, 21]. The expression of *miR-20a* is elevated during osteogenic differentiation, and overexpression of *miR-20a* promotes hMSC osteogenic differentiation by targeting PPAR γ , BAMBI, and CRIM1 [34]. *miR-194* regulates MSC osteogenic differentiation, but inhibits adipocyte differentiation by directly targeting COUP-TFII [35]. In addition, by exerting post-transcriptional control of their specific target genes, miRNAs could regulate osteogenesis through multiple signaling pathways, such as bone morphogenic protein, Notch, Wnt, and transforming growth factor- β signaling pathways [21]. In our previous study, we used vertebral samples of GIOP to perform miRNA-seq analysis and qRT-PCR validation. Using this strategy, we identified nine differentially expressed miRNAs, of which six (*hsa-mir-186-5p*, *hsa-mir-21-5p*, *hsa-mir-214-5p*, *hsa-mir-10b-5p*, *hsa-mir-451a*, and *hsa-mir-novel-chr3_49413*) were significantly upregulated and three (*hsa-let-7f-5p*, *hsa-let-7a-5p*, and *hsa-mir-27a-3p*) were significantly downregulated (Fig. 7). Additionally, the important role of *Let-7f* in osteogenic differentiation has been reported in previous studies [22-25], so we continuously selected *Let-7f* to explore the underlying molecular mechanism of PTE in promoting BMSC osteogenic differentiation. For the first time, the present study showed that the expression of *Let-7f-5p* is significantly

elevated during BMSC osteogenic differentiation induced by PTE, OI, and PTE+OI. Our luciferase assays results further confirmed that *Let-7f-5p* directly targeted the 3' UTR of *Tnfr2* in 293T cells (Fig. 7). This study was limited in that we did not investigate whether knockdown or knockout of *Let-7f-5p* affects the TNFR2/PI3K/AKT signaling pathway or proliferation/osteogenic differentiation in PTE-treated BMSCs, *in vitro* or *in vivo*. Further studies investigating the molecular mechanism will be required to reveal the relationships among *Let-7f-5p*, TNFR2/PI3K/AKT and BMSC osteogenic differentiation.

In this study, we demonstrated that PTE is a positive regulator of BMSC proliferation and osteogenic differentiation. The underlying mechanism involves the upregulation of *Let-7f-5p* miRNA expression levels and inhibition of the signal transduction of the TNFR2/PI3K/AKT pathway. Importantly, our results showed a direct relationship between *Let-7f-5p* and *Tnfr2*, suggesting that PTE may promote BMSC proliferation and osteogenic differentiation. The mechanism of action may be associated with the regulation of *Let-7f-5p* and the TNFR2/PI3K/AKT signaling pathway. A deeper knowledge of this regulatory network may lead to the development of novel therapeutic approaches to enhance bone formation during pathological bone loss.

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Disclosure Statement

The authors declare to have no conflict of interests.

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