Puerarin promotes the proliferation and differentiation of MC3T3-E1 cells via microRNA-106b by targeting receptor activator of nuclear factor-κB ligand

ZIMEI SHAN, NA CHENG, RONG HUANG, BIN ZHAO and YALI ZHOU

Department of Health Care for Cadres, People's Hospital of Xinjiang Uyger Autonomous Region, Urumqi, Xinjiang 830001, P.R. China

Received June 11, 2016; Accepted June 8, 2017

DOI: 10.3892/etm.2017.5405

Abstract. Puerarin, an isoflavone-C-glucoside extracted from the root of *Pueraria Labata* (Willd.) Ohwi, is one of the most important crude herbs used in Chinese medicine for various medicinal purposes. Accumulating evidence has indicated that puerarin suppresses bone resorption and promotes bone formation. However, the molecular mechanism involved in puerarin-associated bone formation is unclear. The present study aimed to investigate the molecular mechanism of puerarin-induced osteoblast proliferation and differentiation. The study showed that puerarin treatment differentially affected cell proliferation in a time-dependent manner. Notably, at a concentration of 20 μM, puerarin significantly promoted cell proliferation in comparison with the control (P<0.01). Furthermore, puerarin promoted MC3T3-E1 cell differentiation at an appropriate concentration. In addition, miR-106b was significantly upregulated in MC3T3-E1 cells following treatment with 20 μM puerarin (P<0.01), and a known target for miR-106b, receptor activator of nuclear factor-κB ligand (RANKL) was demonstrated using the luciferase reporter assay. Furthermore, inhibition of miR-106b significantly reversed the promotion of cell differentiation induced by puerarin in MC3T3-E1 cells (P<0.01). In conclusion, the present study demonstrated that puerarin exerts its role in MC3T3-E1 osteogenesis through miR-106b by targeting RANKL. The findings suggest that puerarin may be considered a promising anti-osteoporotic agent for the treatment of osteoporosis.

Correspondence to: Dr Yali Zhou, Department of Health Care for Cadres, People's Hospital of Xinjiang Uyger Autonomous Region, 28 Chenggong Road, Urumqi, Xinjiang 830001, P.R. China
E-mail: zhou__yali@163.com

Key words: puerarin, microRNA 106b, MC3T3-E1 cell, receptor activator of nuclear factor-κB ligand, osteoporosis

Introduction

Osteoporosis is a systemic disease characterized by reduction of bone tissue content in a unit volume and degeneration of bone microstructure, which increases the risk of fragility fractures (1). Osteoporosis has become a serious social problem as the aging population increases (2). Among the patients affected by osteoporosis, post-menopausal women are disproportionately affected, accounting for >70% of the overall patients (3). The imbalance between bone formation and bone resorption has become an important factor for the formation of osteoporosis (4). Therefore, it is necessary to identify an effective therapeutic agent for the treatment of osteoporosis.

The application of traditional Chinese medicine in China dates back thousands of years and therefore has a high degree of recognition among the population (5). Puerarin is a major bioactive component extracted from the root of *Pueraria Labata* (Willd.) Ohwi, also known as Kudzu, and is one of the earliest medicinal plants to be used in China. (6). Puerarin displays a series of properties that attenuate osteoporosis, inflammation and liver injury (7). In osteoporosis studies, puerarin has been demonstrated to reduce bone reabsorption, promoting bone formation and increasing bone mineral density (8,9). Therefore, it is important to explore the therapeutic effects and underlying mechanisms of puerarin on osteoporosis.

MicroRNAs (miRNAs or miRs) are endogenous, non-coding, single-stranded small RNAs with a length of ~22 nucleotides, which are widespread in eukaryotic genomes (10). miRNAs function in the post-transcriptional regulation of gene expression by binding to the 3'-untranslated regions (UTRs) of target mRNAs (10,11), which leads to translation inhibition or degradation of mRNA (12). miRNAs are known to have a significant role in biological processes, including cell proliferation (13,14), apoptosis (15,16), differentiation (17,18) and tumor formation (19). Recent studies suggest that miRNAs may also be associated with bone formation and osteoblast differentiation (20-22). For example, Wang *et al* (23) identified that microRNA-106b inhibited osteolysis by targeting receptor activator of nuclear factor-κB ligand (RANKL) in giant cell tumors in the bone. However, whether miR-106b participates in the anti-osteoporotic effects of puerarin is currently unknown.

The aim of the present study was to explore the effects and related mechanisms of puerarin on the prevention of osteoporosis in MC3T3-E1 cells. Based on the important role of miR-106b in osteoporosis, it was investigated whether miR-106b is associated with the anti-osteoporotic effect of puerarin.

Materials and methods

Cell lines, chemicals and biochemical reagents. The MC3T3-E1 cell line, which is an osteoblast-like cell line from C57BL/6 mouse calvaria, was obtained from American Type Culture Collection (CRL-2593; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat inactivated fetal bovine serum (Sigma-Aldrich, Merck KGaA; Darmstadt, Germany) 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 37˚C in an atmosphere containing 5% CO_2. Puerarin was purchased from the National Institutes for Food and Drug Control (Beijing, China). The assay kit for alkaline phosphatase (ALP; cat. no. A059-2) was obtained from Nanjing Jiancheng Biotechnology Institute Co., Ltd. (Nanjing, China). ELISA kits for type 1 collagen (COL I; cat. no. DY6220-05) and osteocalcin (OCN; cat. no. QC137) assays were purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

MTT assay. Cell proliferation and growth was determined using an MTT assay according to the manufacturer's instructions. A total of ~2x10^4 cells/well were seeded in a 96-well culture plate for 24 h at 37˚C and treated with different concentrations (0, 5, 10, 20 or 40 μM) of puerarin for 1, 2, 3, 4 and 5 days at 37˚C in a humidified incubator with 5% CO_2. Non-treated cells served as the control. Subsequently, 0.5 mg/ml MTT solution (Sigma-Aldrich; Merck KGaA) was added to each well and incubated at 37˚C for 3 h. Following the removal of the culture medium, the cells were washed twice with PBS and 100 μl of 0.01% HCl-isopropanol solution was added to dissolve the formazan crystals. Absorbance was recorded at 490 nm using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

COL I and OCN content and ALP activity. COL I and OCN content of MC3T3-E1 cells were measured as described previously (24,25). A total of ~1.0x10^5 cells in 1 ml DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin were added to each well at 37˚C for 24 h following the addition of diluted puerarin (5, 10, 20 or 40 μM). After 7 and 14 days, the COL I and OCN contents were determined using the ELISA assay kit. Absorbance was recorded at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.). The supernatant was used for ALP activity determination after 3, 5 and 7 days of incubation with ALP assay kits. Untreated MC3T3-E1 cells served as controls.

Mineralization assay (Alizarin Red-S staining). MC3T3-E1 cells (1x10^5 cells/well) were seeded into a 12-well plate. After 14 days of puerarin treatment (5-40 μM) at 37˚C, the supernatant was removed and the cells were fixed with 4% neutral formaldehyde in PBS at room temperature for 10 min and the cells were washed twice with distilled water. Subsequently, cells were stained with 0.1% (w/v) Alizarin Red-S (pH 4.2; Sigma-Aldrich; Merck KGaA) at 37˚C for 30 min (26). Following staining, the cultures were washed thoroughly with deionized water and the absorbance was recorded on a microplate reader (Bio-Rad Laboratories, Inc.) at 562 nm.

Transfection. The miR-106b mimics (5'-TAAAGTGCTGACAGTGCAGAT-3'), mimics negative control (mimics NC; 5'-UUGUACUACACAAAAGUACUG-3'), miR-106b inhibitors (5'-ATCTGCACTGTCAGCACTTTA-3') and inhibitor NC (5'-TTCTCCGAACGTGTCACGT-3') were purchased from RiboBio Co., Ltd. (Guangzhou, China). MC3T3-E1 cells were transfected with oligonucleotides (50 pmol/ml) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cell lysates were harvested at 48 h after transfection. A total of 50 nM miR-106b inhibitors or inhibitor NC were transfected into MC3T3-E1 cells then induced with peurarin for 3, 5, 7 or 14 days. Following induction, the supernatant was used for COL I, OCN and ALP analyses (on 7 and 14 days) and the cells (on days 3, 5, 7) were used for ALP activity determination using ALP assay kits.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA from MC3T3-E1 cells was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was synthesized from 2 μg of total RNA using PrimeScript RT Reagent kit (cat. no. DRR037A; Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. The reverse transcription step consisted of an incubation at 50˚C for 5 min. qPCR anaylsis of miR-106b was performed using the TaqMan miRNA assay RT-PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The specific primers were synthesized commercially from Sangon Biotech Co., Ltd. (Shanghai, China) as follows: miR-106b, forward 5'-TAAAGTGCTGACAGTGCAGATAGTG-3', and reverse 5'-CAAGTACCCACAGTGCGGT-3', and U6, forward 5'-CTCGCTTCGGCAGCACA-3', and reverse 5'-AACGCTTCACGAATTTGCGT-3'. The PCR conditions were as follows: Initial denaturation at 95˚C for 10 min followed by 40 cycles consisting of 95˚C for 5 sec, 60˚C for 30 sec and 72˚C for 10 sec. The fluorescence signal was monitored at 585 nm during each extension. Data were analyzed using 7500 software version 2.0.1 (Applied Biosystems; Thermo Fisher Scientific, Inc.), and calculated using the $2^{-\Delta\Delta Cq}$ method (27), with U6 small nuclear RNA as the endogenous control. PCR reactions were performed using QuantiTect SYBR Green PCR Master mix (Qiagen, Inc., Valencia, CA, USA) in an Applied Biosystems 7500 instrument according to the manufacturer's protocol.

Luciferase reporter assay. The 3'-UTR of RANKL with wild-type (WT) or mutant (Mut) binding sites for miR-106b was amplified and subcloned into the pGL3 vector (Promega Corporation, Madison, WI, USA) to generate the plasmid pGL3-WT-RANKL-3'-UTR or pGL3-Mut-RANKL-3'-UTR, respectively. The HEK293 cells were seeded in 24-well plate in duplicate (5x10^4 cell/well), when the cells reached 70% confluence they were co-transfected with 1-2 μg/ml of either

pGL3-WT-RANKL-3'-UTR or pGL3-Mut-RANKL-3'-UTR plasmids and 25 nM miR-106b mimics and miR-106b inhibitor using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. Mimics and inhibitors NC were transfected using the same procedure. The pRL-TK plasmid (Promega Corporation) was used as a normalizing control. After 48 h of incubation, luciferase activity was analyzed using the Dual-Luciferase® Reporter Assay System (Promega Corporation).

Western blot analysis. MC3T3-E1 cells were lysed on ice with RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) and protein concentrations were determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). A total of 40 μg/lane protein was separated by 10% SDS-PAGE (Bio-Rad Laboratories, Inc.) and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA), which was incubated with 5% fat-free skim milk in TBS containing 0.05% Tween-20 for 1 h at room temperature. The blots were then incubated overnight at 4˚C with anti-RANKL (Rabbit monoclonal; 1:1,000; cat. no. ab45039) and anti-β-actin (Rabbit monoclonal; 1:1,000; cat. no. ab32572) primary antibodies (both Abcam, Cambridge, MA, USA). The blots were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (monoclonal; 1:10,000; cat. no. ab99702; Abcam) for 1 h at room temperature. An enhanced chemiluminescence substrate (EMD Millipore) was used to visualize signals. β-actin was used as an endogenous protein for normalization. Relative band intensities were determined by densitometry using Quantity One 4.6.2 software (Bio-Rad Laboratories, Inc.).

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (version 5.0; GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as the mean ± standard deviation as indicated. One-way analysis of variance followed by a Tukey's post hoc test or two-tailed Student's t-test was used for comparisons between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Growth promotion of MC3T3-E1 cells induced by puerarin. To investigate the effect of puerarin on the growth of MC3T3-E1 cells, the MTT assay was performed to measure cell growth of MC3T3-E1 cells following treatment with various concentrations (0-40 μM) of puerarin for 1, 2, 3, 4 and 5 days. As indicated in Fig. 1, the proliferation activity of MC3T3-E1 cells was markedly promoted by puerarin in a time- and dose-dependent manner, with the peak level at 20 μM. Based on the dose dependent curves, 20 μM was chosen as the optimal dosage for consequent experiments.

Effect of puerarin on osteoblast differentiation. To clarify the role of puerarin in osteoblast differentiation, MC3T3-E1 cells were incubated with different concentrations of puerarin. As shown in Fig. 2A, ALP activity was increased by puerarin treatment in a time-dependent manner up to a concentration of 20 μM, after which the activity was reduced. ALP activity was significantly increased in the 20 and 40 μM puerarin treatment groups at 3 (P<0.01 and P<0.05, respectively), 5 (P<0.01) and 7 (P<0.01) days compared with the control group, and 10 μM puerarin treatment at 7 days also exhibited a significant increase in ALP activity compared with the control (P<0.05). COL I and OCN content was significantly increased in a dose-dependent manner at 7 and 14 days of culture compared with the control group with 20 and 40 μM puerarin treatment (P<0.01), although both were markedly reduced following 40 μM treatment in comparison with 20 μM (Fig. 2B and C). Furthermore, mineralization was significantly increased after 14 days of culture with 10 (P<0.05), 20 (P<0.01) or 40 μM (P<0.01) puerarin treatment (Fig. 2D). In addition, these results indicated that the optimal concentration of puerarin effect on cell differentiation was 20 μM. Furthermore, these data indicated that puerarin may promote osteoblast differentiation.

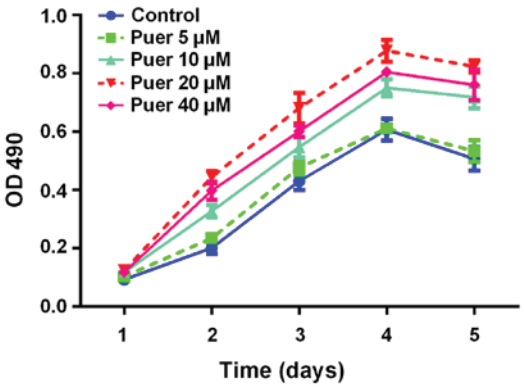

Figure 1. Effect of puerarin on cell proliferation in MC3T3-E1 cells. Cells were treated with puerarin (0, 5, 10, 20 or 40 μM) for 1-5 days and cell growth was measured by MTT assay. Data are presented as the mean ± standard deviation of three independent experiments. Puer, puerarin; OD, optical density.

Effect of puerarin on the expression of miR-106b. It has been recently reported that miR-106b may inhibit osteoclastogenesis and osteolysis in giant cell tumors of bone (20). Therefore, it was assumed that puerarin may induce the differentiation of MC3T3-E1 cells by upregulating the expression of miR-106b. The expression levels of miR-106b were determined by RT-qPCR following treatment with puerarin in MC3T3-E1 cells for 48 h. Fig. 3A showed that 10, 20 and 40 μM puerarin treatment significantly increased the expression levels of miR-106b in MC3T3-E1 cells compared with the control (P<0.05, P<0.01 and P<0.01, respectively).

A recent study demonstrated that miR-106b inhibited osteoclastogenesis and osteolysis by suppressing the expression of RANKL (28). However, whether puerarin was able to promote the proliferation and differentiation of MC3T3-E1 cells through miR-106b by targeting RANKL is unknown. To determine whether miR-106b directly targets RANKL, the direct binding of miR-106b to RANKL mRNA 3'-UTR was investigated using the luciferase report assay (Fig. 3B). According to RT-qPCR analysis, a significant decrease in miR-106b expression following transfection of miR-106b inhibitor was indicated compared with the control (P<0.01; Fig. 3C), and a 6.5-fold significant increase of mature miR-106b expression was revealed in MC3T3-E1 cells at 24-h post-transfection of miR-106b mimics compared with

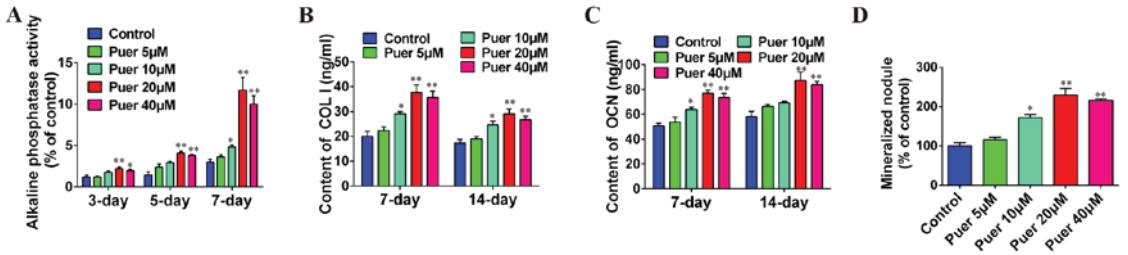

Figure 2. Puerarin promoted cell differentiation in MC3T3-E1 cells. MC3T3-E1 cells were incubated with various concentrations (0, 5, 10, 20 or 40 μM) of puerarin for 3, 5, 7 or 14 days. Medium was collected for COL I and OCN content (at 7 and 14 days) and ALP activity (at 3, 5 and 7 days) determination using ELISA kits. Medium was collected for Alizarin red staining at 14 days. (A) Effect of puerarin on alkaline phosphatase activity, (B) COL I secretion, and (C) OCN secretion of MC3T3-E1 cells, and (D) bone mineralization of MC3T3-E1 cells. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. control. Puer, puerarin; COL I, collagen type 1; OCN, osteocalcin.

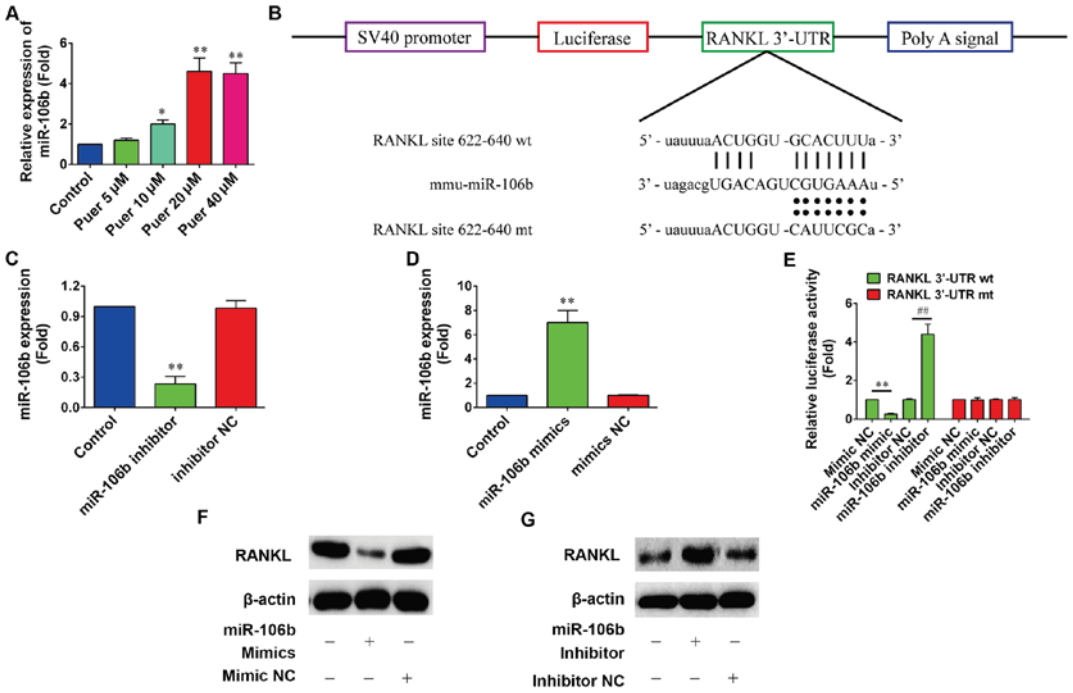

Figure 3. Puerarin promoted the expression of miR-106b. (A) Expression levels of miR-106b were detected by reverse transcription-quantitative polymerase chain reaction in MC3T3-E1 cells following puerarin treatment for 72 h. (B) Schema of the firefly luciferase reporter constructs for RANKL, which indicated the interaction sites between miR-106b and the 3'-UTRs of the RANKL. (C and D) Expression levels of miR-106b following treatment with miR-106b mimic or miR-106b inhibitor were determined (n=6). *P<0.05 and **P<0.01 vs. control. (E) Dual-luciferase reporter assay detected the interaction between miR-106b and the 3'-UTR of RANKL. miR-106b mimic, mimic NC, miR-106b inhibitor or inhibitor NC were co-transfected with pGL-RANKL reporter vectors into HEK293 cells for 48 h. (n=6). **P<0.01 vs. mimics NC, $^{\#\#}$P<0.01 vs. inhibitor NC. (F and G) Protein expression levels of RANKL following treatment with miR-106b mimic or miR-185 inhibitor were indicated (n=6). All data are presented as the mean ± standard deviation results of three independent experiments. miR-106b, microRNA-106b; RANKL, receptor activator of nuclear factor-κB ligand; UTR, untranslated region; NC, negative control; mt, mutant; wt, wild-type.

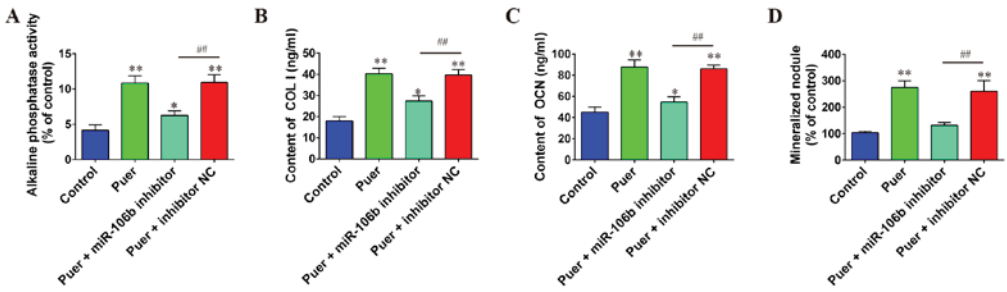

Figure 4. Inhibition of miR-106b reversed osteogenic differentiation induced by puerarin. MC3T3-E1 cells were transfected with miR-106b inhibitor or NC prior to treatment with puerarin. Effect of miR-106b inhibition on (A) alkaline phosphatase activity (B) COL I secretion, (C) OCN secretion and (D) bone mineralization of MC3T3-E1 cells treated with puerarin. All data were represented as the mean ± standard deviation of three independent experiments. *P<0.05, **P<0.01 vs. control, $^{\#\#}$P<0.01 vs. Puer+inhibitor NC. Puer, puerarin; miR-106b, microRNA-106b; COL I, collagen type 1; OCN, osteocalcin; NC, negative control.

the control (P<0.01; Fig. 3D). The luciferase results showed that overexpression of miR-106b significantly decreased (P<0.01) the luciferase activity in pGL3-RANKL 3'-UTR wild-type transfected cells, whereas it had no significant effect on pGL3 RANKL 3'-UTR mutant cells (Fig. 3E). Furthermore, western blot analysis revealed that miR-106b overexpression markedly decreased the protein expression level of RANKL (Fig. 3F), whereas miR-106b inhibition markedly increased the protein expression of RANKL (Fig. 3G).

Effect of miR-106b on osteogenic differentiation induced by puerarin. To explore the role of miR-106b during osteogenic differentiation induced by puerarin, miR-106b inhibitor and respective NC were co-transfected into MC3T3-E1 cells, following puerarin treatment. Results indicated that knock-down of miR-106b significantly decreased differentiation and mineralization of MC3T3-E1 pre-osteoblastic cells caused by puerarin as evidenced by the decreased contents or activities of major markers COL I (P<0.01), OCN (P<0.01), ALP (P<0.01) and mineral nodule formation (P<0.01) compared to the puer+inhibitor NC group (Fig. 4). These results indicated that the elevation of miR-106b may be associated with the process of puerarin-induced differentiation in MC3T3-E1 cells.

Discussion

In the present study, it was indicated that puerarin promoted the growth of MC3T3-E1 cells in a time- and dose-dependent manner up to a concentration of 20 μM. Furthermore, these findings have provided evidence that puerarin may promote the differentiation of MC3T3-E1 cells through miR-106b by targeting RANKL. The present work revealed the anti-osteoporotic effects of puerarin in MC3T3-E1 cells, suggested the participation of miR-106b in this process and thus indicated that puerarin may be a novel agent for treatment of osteoporosis.

Several reports have demonstrated that puerarin is able to promote osteoblast proliferation and differentiation (29,30). A study from Wong and Rabie (31) showed that puerarin treatment may prevent bone loss in a dose-dependent manner. Similarly, Urasopon *et al* (32) showed that in cultures of newborn Wistar rat osteoblasts, puerarin was also effective in stimulating osteoblastic bone formation. Thus, it is important to understand the underlying mechanisms of this effect. Sheu *et al* (33) previously discovered that puerarin-mediated osteoblast proliferation is likely to be mediated by bone morphogenetic proteins and nitric oxide (NO) pathways in adult female mouse osteoblasts. In agreement with these studies, the present data reinforced that puerarin affects the induction of osteoblast proliferation and differentiation.

Previous studies have reported that miRNAs were associated with osteogenesis (34,35). For example, in human adipose tissue-derived stem cells, mouse mesenchymal ST2 stem cells and mouse premyogenic C2C12 cells, several miRNAs (miR-26a, -125b, -133 and -135) have been reported to regulate osteoblast cell growth or differentiation (36). A study performed by Shi *et al* (37) demonstrated that in C2C12 cells under osteogenic differentiation, miR-214 has an important role as a suppressor by targeting osterix. However, it is unknown whether miRNAs mediate the anti-osteoporotic effect of puerarin.

A variety of studies have demonstrated that miR-106b participates in the progression of osteoporosis (38,39). For example, miR-106b expression levels may enhance osteoclast differentiation and bone resorption by targeting RANKL, twist-related protein (TWIST) and matrix metallopeptidase (MMP)2, which may partly elucidate the role of miR-106b downregulation in giant cell tumors and bone metastasis (23). Previous studies have suggested that various target genes of miR-106b, including interleukin-8 (40), MMP2 (41) and TWIST (42), have been reported to induce cell proliferation and invasion in cancers. Zheng *et al* (43) identified that miR-106b induced epithelial-mesenchymal transition by targeting paired related homeobox 1 in colorectal cancer. The present data indicated that puerarin promoted the expression of miR-106b, which mediated the anti-osteoporotic effect of puerarin in MC3T3-E1 cells via regulating RANKL expression levels.

In conclusion, the present study investigated the potential functions of puerarin in cell growth and differentiation in MC3T3-E1 cells. The present findings suggested that puerarin positively affected osteogenic differentiation through the upregulation of miR-106b by directly targeting RANKL. Therefore, the present results indicate a novel mechanism for puerarin-induced promotion of bone formation activity, which may be used as a therapeutic target for the treatment of osteoporosis.

References

1. Wirries A, Schubert AK, Zimmermann R, Jabari S, Ruchholtz S and El-Najjar N: Thymoquinone accelerates osteoblast differentiation and activates bone morphogenetic protein-2 and ERK pathway. Int Immunopharmacol 15: 381-386, 2013.
2. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, *et al*: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352: 987-996, 2005.
3. Johnell O and Kanis JA: An estimate of the worldwide prevalence and disability associated with osteoporotic fractures. Osteoporos Int 17: 1726-1733, 2006.
4. Rodan GA and Martin TJ: Therapeutic approaches to bone diseases. Science 289: 1508-1514, 2000.
5. Liu Y, Liu JP and Xia Y: Chinese herbal medicines for treating osteoporosis. Cochrane Database Syst Rev: CD005467, 2014.
6. Keung WM, Lazo O, Kunze L and Vallee BL: Potentiation of the bioavailability of daidzin by an extract of Radix puerariae. Proc Natl Acad Sci USA 93: 4284-4288, 1996.
7. Maji AK, Pandit S, Banerji P and Banerjee D: Pueraria tuberosa: A review on its phytochemical and therapeutic potential. Nat Prod Res 28: 2111-2127, 2014.
8. Persson I, Weiderpass E, Bergkvist L, Bergström R and Schairer C: Risks of breast and endometrial cancer after estrogen and estrogen-progestin replacement. Cancer Causes Control 10: 253-260, 1999.
9. Orija IB and Mehta A: Hormone replacement therapy: Current controversies. Clin Endocrinol (Oxf) 59: 657, 2003.
10. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
11. Carthew RW and Sontheimer EJ: Origins and mechanisms of miRNAs and siRNAs. Cell 136: 642-655, 2009.
12. Erson AE and Petty EM: MicroRNAs in development and disease. Clin Genet 74: 296-306, 2008.
13. Laneve P, Di Marcotullio L, Gioia U, Fiori ME, Ferretti E, Gulino A, Bozzoni I and Caffarelli E: The interplay between microRNAs and the neurotrophin receptor tropomyosin-related kinase C controls proliferation of human neuroblastoma cells. Proc Natl Acad Sci USA 104: 7957-7962, 2007.
14. Wang X, Tang S, Le SY, Lu R, Rader JS, Meyers C and Zheng ZM: Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. PLoS One 3: e2557, 2008.

15. Song G, Zhang Y and Wang L: MicroRNA-206 targets notch3, activates apoptosis, and inhibits tumor cell migration and focus formation. J Biol Chem 284: 31921-31927, 2009.
16. Thompson BJ and Cohen SM: The Hippo pathway regulates the bantam microRNA to control cell proliferation and apoptosis in Drosophila. Cell 126: 767-774, 2006.
17. Li X and Carthew RW: A microRNA mediates EGF receptor signaling and promotes photoreceptor differentiation in the Drosophila eye. Cell 123: 1267-1277, 2005.
18. Kawasaki H and Taira K: Retraction: Hes1 is a target of microRNA-23 during retinoic-acid-induced neuronal differentiation of NT2 cells. Nature 426: 100, 2003.
19. Foekens JA, Sieuwerts AM, Smid M, Look MP, de Weerd V, Boersma AW, Klijn JG, Wiemer EA and Martens JW: Four miRNAs associated with aggressiveness of lymph node-negative, estrogen receptor-positive human breast cancer. Proc Natl Acad Sci USA 105: 13021-13026, 2008.
20. Arfat Y, Xiao WZ, Ahmad M, Zhao F, Li DJ, Sun YL, Hu L, Zhihao C, Zhang G, Iftikhar S, *et al*: Role of microRNAs in osteoblasts differentiation and bone disorders. Curr Med Chem 22: 748-758, 2015.
21. Papaioannou G, Mirzamohammadi F and Kobayashi T: MicroRNAs involved in bone formation. Cell Mol Life Sci 71: 4747-4761, 2014.
22. Pi C, Li YP, Zhou X and Gao B: The expression and function of microRNAs in bone homeostasis. Front Biosci 20: 119-138, 2015.
23. Wang T, Yin H, Wang J, Li Z, Wei H, Liu Z, Wu Z, Yan W, Liu T, Song D, *et al*: MicroRNA-106b inhibits osteoclastogenesis and osteolysis by targeting RANKL in giant cell tumor of bone. Oncotarget 6: 18980-18996, 2015.
24. Horiguchi Y, Nakai T and Kume K: Effects of Bordetella bronchiseptica dermonecrotic toxin on the structure and function of osteoblastic clone MC3T3-e1 cells. Infect Immun 59: 1112-1116, 1991.
25. James AW: Review of signaling pathways governing MSC osteogenic and adipogenic differentiation. Scientifica (Cairo) 2013: 684736, 2013.
26. Tang X, Lin J, Wang G and Lu J: MicroRNA-433-3p promotes osteoblast differentiation through targeting DKK1 expression. PLoS One 12: e0179860, 2017.
27. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
28. Wang Y, Yang C, Xie WL, Zhao YW, Li ZM, Sun WJ and Li LZ: Puerarin concurrently stimulates osteoprotegerin and inhibits receptor activator of NF-κB ligand (RANKL) and interleukin-6 production in human osteoblastic MG-63 cells. Phytomedicine 21: 1032-1036, 2014.
29. Lv H, Che T, Tang X, Liu L and Cheng J: Puerarin enhances proliferation and osteoblastic differentiation of human bone marrow stromal cells via a nitric oxide/cyclic guanosine monophosphate signaling pathway. Mol Med Rep 12: 2283-2290, 2015.
30. Wang C, Meng MX, Tang XL, Chen KM, Zhang L, Liu WN and Zhao YY: The proliferation, differentiation, and mineralization effects of puerarin on osteoblasts in vitro. Chin J Nat Med 12: 436-442, 2014.
31. Wong R and Rabie B: Effect of puerarin on bone formation. Osteoarthritis Cartilage 15: 894-899, 2007.
32. Urasopon N, Hamada Y, Cherdshewasart W and Malaivijitnond S: Preventive effects of Pueraria mirifica on bone loss in ovariectomized rats. Maturitas 59: 137-148, 2008.
33. Sheu SY, Tsai CC, Sun JS, Chen MH, Liu MH and Sun MG: Stimulatory effect of puerarin on bone formation through co-activation of nitric oxide and bone morphogenetic protein-2/mitogen-activated protein kinases pathways in mice. Chin Med J (Engl) 125: 3646-3653, 2012.
34. Li Z, Hassan MQ, Volinia S, van Wijnen AJ, Stein JL, Croce CM, Lian JB and Stein GS: A microRNA signature for a BMP2-induced osteoblast lineage commitment program. Proc Natl Acad Sci USA 105: 13906-13911, 2008.
35. Kapinas K, Kessler C, Ricks T, Gronowicz G and Delany AM: miR-29 modulates Wnt signaling in human osteoblasts through a positive feedback loop. J Biol Chem 285: 25221-25231, 2010.
36. Itoh T, Nozawa Y and Akao Y: MicroRNA-141 and -200a are involved in bone morphogenetic protein-2-induced mouse pre-osteoblast differentiation by targeting distal-less homeobox 5. J Biol Chem 284: 19272-19279, 2009.
37. Shi K, Lu J, Zhao Y, Wang L, Li J, Qi B, Li H and Ma C: MicroRNA-214 suppresses osteogenic differentiation of C2C12 myoblast cells by targeting Osterix. Bone 55: 487-494, 2013.
38. Collison J: Bone: miR-106b promotes osteoporosis in mice. Nat Rev Rheumatol 13: 130, 2017.
39. Liu K, Jing Y, Zhang W, Fu X, Zhao H, Zhou X, Tao Y, Yang H, Zhang Y, Zen K, *et al*: Silencing miR-106b accelerates osteogenesis of mesenchymal stem cells and rescues against glucocorticoid-induced osteoporosis by targeting BMP2. Bone 97: 130-138, 2017.
40. Chuang TD, Luo X, Panda H and Chegini N: miR-93/106b and their host gene, MCM7, are differentially expressed in leiomyomas and functionally target F3 and IL-8. Mol Endocrinol 26: 1028-1042, 2012.
41. Ni X, Xia T, Zhao Y, Zhou W, Wu N, Liu X, Ding Q, Zha X, Sha J and Wang S: Downregulation of miR-106b induced breast cancer cell invasion and motility in association with overexpression of matrix metalloproteinase 2. Cancer Sci 105: 18-25, 2014.
42. Dong P, Kaneuchi M, Watari H, Sudo S and Sakuragi N: MicroRNA-106b modulates epithelial-mesenchymal transition by targeting TWIST1 in invasive endometrial cancer cell lines. Mol Carcinog 53: 349-359, 2014.
43. Zheng L, Zhang Y, Lin S, Sun A, Chen R, Ding Y and Ding Y: Down-regualtion of miR-106b induces epithelial-mesenchymal transition but suppresses metastatic colonization by targeting Prrx1 in colorectal cancer. Int J Clin Exp Pathol 8: 10534-10544, 2015.