Puerarin promotes the viability and differentiation of MC3T3-E1 cells by enhancing LC3B-mediated autophagy through downregulation of miR-204

QIAN FENG[1*], SONG-YI CHENG[2*], RUI YANG[1], XIANG-WEI ZENG[3], FENG-MING ZHAO[1] and XIU-QIN ZHAN[1]

[1]School of Medicine and Life Sciences, Nanjing University of Chinese Medicine, Nanjing, Jiangsu 210023;
[2]Department of Cardiology, Nanjing Hospital of Chinese Medicine, Nanjing, Jiangsu 210001;
[3]School of Life Sciences, Nanjing University, Nanjing, Jiangsu 210023, P.R. China

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Abstract. Puerarin is a bioactive substance extracted from *Pueraria lobata*. It is known to promote the viability, differentiation and mineralization of osteoblasts. However, the molecular mechanisms involved in these activities are not well understood. The present study was conducted with the aim of elucidating the effect of puerarin on osteoblasts and to explore the underlying mechanism. CCK-8 analysis showed that puerarin (0.1, 1 and 10 μM) promoted the viability of osteoblastic MC3T3-E1 cells, with 1 μM of puerarin exhibiting the strongest effect. Moreover, 1 μM puerarin significantly increased the activity of alkaline phosphatase (ALP) and the formation of mineralized nodules in the MC3T3-E1 cells. Treatment with 1 μM puerarin for 72 h led to a significant upregulation in the expression level of microtubule-associated light chain 3 (LC3)B and Beclin1 proteins. This treatment was more effective in promoting LC3B expression than what was observed following treatment with rapamycin (overexpression for autophagy). The bilayer membrane structure of autophagosomes was observed by electron microscopy. Conversely, 3-methyladenine (3-MA, inhibitor of autophagy) reduced the cell viability as well as the activity of alkaline phosphatase (ALP) in MC3T3-E1 cells, although, there was no significant influence on mineralization. Prediction results of the biological information showed that LC3B could be a direct target of microRNA-204 (miR-204). In the present study, the expression level of miR-204 was decreased by puerarin. miR-204 mimics significantly decreased LC3B expression and inhibited auotophagosome formation, while the miR-204 inhibitor had the opposite effects. To conclude, the results of the present study suggest that puerarin promotes the viability and differentiation of MC3T3-E1 cells through autophagy, which is possibly associated with miR-204-regulated LC3B upregulation.

Correspondence to: Professor Xiu-Qin Zhan, School of Medicine and Life Sciences, Nanjing University of Chinese Medicine, 138 Xianlin Avenue, Nanjing, Jiangsu 210023, P.R. China
E-mail: xqzhan14@sina.com

*Contributed equally

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Introduction

Osteoporosis is a systemic osteopathy. It results in a decrease in both the density and mass of bones, ultimately causing bone microstructure damage and increased bone fragility (1). Osteoblasts are crucial for the process of bone formation. Defects in osteoblast viability, differentiation and mineralization are among the major causes of osteoporosis (2,3). Puerarin, an isoflavone extracted from the Chinese medicine pueraria, exhibits protective functions on the cardiovascular system, nervous system, osteoporosis, liver injury, and inflammation *in vivo* and *in vitro* (4). Studies have shown that puerarin can promote the proliferation, differentiation and mineralization of osteoblasts *in vitro* (5,6). In a recent report, puerarin was found to improve bone loss in estrogen-deficient rats, display a superior anti-osteoporosis effect, and prevent osteoporosis in postmenopausal women (7). Although experiments have shown that puerarin plays an important role in osteoblasts and osteoporosis *in vitro* and *in vivo*, the exact mechanisms involved in the anti-osteoporosis effects of puerarin remain unclear.

There are three main types of cell death: Autophagy, apoptosis and necrosis (8). The level of autophagy changes significantly during the process of osteoporosis. Moreover, autophagy can regulate osteoblasts in both directions to either promote or inhibit cell proliferation under different conditions (9). Cells can generate energy from the degradation pathway of autophagy. But if the damage is exceedingly high, the cells will undergo controlled suicide by autophagy (10). The above findings warrant further investigation of the effect of puerarin on autophagy in osteoblasts.

Microtubule-associated light chain 3 (LC3) exists in the double membrane structure of autophagosomes, and is an important marker of autophagy. There are three isoforms of LC3 proteins in mammals: LC3A, LC3B and LC3C.

These three undergo post-translational modifications during autophagy (11). The LC3 protein is immediately synthesized by Atg4 at the carboxyl end of the protein, and LC3-I is localized in the cytoplasm. We discovered that during autophagy, LC3-I is modified and processed by ubiquitin-like systems, including Atg7 and Atg3. The overall result is LC3-II with molecular weight of 16 kDa localized in autophagic microsomes. Thus, the presence of LC3-I and low molecular weight LC3-II in autophagosome are both molecular markers of autophagy, and the amount of LC3-II represents the degree of autophagy (12). To date, LC3B has been extensively studied and identified as an autophagic membrane-associated factor (13). Previous studies have demonstrated that a low concentration of puerarin can promote the viability and differentiation of osteoblastic MC3T3-E1 cells, which may be achieved by downregulation of the expression of miR-204 and its host gene *TRPM3* (6). However, it is not clear whether there are other targets in this pathway. Biological information indicates that there may be a targeting relationship between miR-204 and LC3B, but this has not been verified in osteoblasts.

In the present study, the effects of puerarin on the viability, differentiation and mineralization of osteoblasts were observed. It was found that puerarin significantly upregulated the expression of LC3B and Beclin1 leading to the formation of autophagosomes in osteoblasts. In addition, inhibition of autophagy in osteoblasts significantly reduced the viability and differentiation of the cells. It is suggested that puerarin enhances the viability and differentiation of osteoblasts by promoting autophagy. The possible binding sites between miR-204 and LC3B were explored based on biological information. In addition, the overexpression of miR-204 significantly reduced the protein level of LC3B and the formation of autophagosomes, while its inhibition reversed this effect. To conclude, puerarin promoted the viability and differentiation of MC3T3-E1 cells through autophagy, a mechanism possibly associated with miR-204-mediated LC3B upregulation.

Materials and methods

Osteoblast culture. Osteoblastic MC3T3-E1 cells were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in a 25 cm^2 flask, and α-modified Eagle's medium (α-MEM; Wisent Inc., Canada) was added together with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) 100 U/ml penicillin and 100 g/ml streptomycin and maintained at 37˚C in a humid 5% CO_2 incubator (Sanyo Electric Co., Ltd., Japan).

CCK-8 assay. Firstly, $5x10^3$ cells/well were seeded in 96-well plates (Corning Inc.) at 37˚C in a humid 5% CO_2 incubator for 24 h, and then 200 μl of different final concentrations (0.1, 1 and 10 μM) of puerarin were added to experimental wells, while equivalent serum-free medium was added to the control cells. After culturing for 24, 48, 72 h, 10 μl CCK-8 (Dojindo Molecular Technologies) was added to each well and culturing was continued for 30 min to detected optical density (OD) values (wavelength 450 nm) using a multi-functional enzyme labeling instrument (BioTek Instruments, Inc.). The cell viability following treatment with 3-MA, or puerarin + 3-MA was also detected by CCK-8 assay.

Alkaline phosphatase (ALP) activity. Cells at a density of $5x10^5$ cells/well were seeded in 6-well plates. Following culture for 24 h, 2 ml of 1 μM puerarin was added to the experimental wells, while equivalent serum-free medium was added to the control cells. Following culturing for 72 h, the cell ALP activity was determined using an ALP kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). This method was also used to detect cell ALP activity of the cells following treatment with 3-MA, or puerarin + 3-MA.

Count of mineralized nodules. A total of $5x10^4$ cells were added to each well of a 24-well culture plate. After 24 h, the cells were treated with puerarin, 3-MA, or puerarin + 3-MA. The medium was changed every 3 days. The cells were washed 2 times with PBS and stained with 0.2% solution of alizarin red for 30 min on day 7, 14 and 21. Three fields were randomly selected for each well under low magnification, and the relative mineralized nodule areas were analyzed by Image J 2x 2.1.4.7 software (Rawak Software Inc.).

Transmission electron microscopy. A total of $5x10^5$ cells/well were seeded in 6-well plates. After culturing for 24 h, 2 ml/well fresh culture medium was added according to the experiment grouping (control group, 1 μM puerarin group, 1 μM puerarin+3-MA group). Following culturing for 72 h, 1 ml/well trypsin was added to digest the cells in a 1.5 ml EP tube, and then the cells were centrifuged at 1,000 x g for 5 min and the supernatant was removed. Next glutaraldehyde (500 ml/tube) was added and the cells were maintained at 4˚C for 12 h, and then osmium acid was used to fix the cells which then underwent uranium acetate staining, ethanol gradient dehydration and resin embedding. Finally, the structure of the cell organelles was observed under a transmission electron microscope (Jeol, Tokyo, Japan; magnification, x12,000). This method was also used to observe cell organelles in the cells transfected with miR-204 NC, miR-204 mimics and miR-204 inhibitor.

Western blot analysis. Cells at a density of $5x10^5$ cells/well were seeded in 6-well plates. After culturing for 24 h, the cells were treated with puerarin for 0, 48 and 72 h. Cell total protein was extracted using a total protein extraction kit (cat. no. BC3710; Beijing Solarbio Science & Technology Co., Ltd., China) and quantified by the BCA method. 12% SDS-PAGE was performed on a 25 g sample, and the separated proteins were electrotransfer onto a polyvinylidene phosphatide membrane, and blocked using 5% skim milk in TBS containing 0.1% Tween-20 (TBST) at room temperature for 1 h. After 1 h, the membranes were washed 3 times with TBST for 15 min. Next, primary antibodies including LC3B (cat. no. ab48394; Abcam), Beclin1 (cat. no. ab62557; Abcam) and β-actin (cat. no. 4970; Cell Signaling Technology, Inc.) were mixed with blocking solution (dilution 1:1,000) separately in 4 ml and used to treat the membranes at 4˚C overnight. The membranes were then washed 3 times with TBST for 15 min, and then incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (cat. no. 7074; Cell Signaling Technology, Inc.; dilution 1:2,000) for 1 h at room temperature. After washing with

TBST, the protein bands of interest were displayed by enhanced chemiluminescence scenario detection system. (Bio-Rad Laboratories, Inc.). ImageJ2x V2.1.4.7 software (National Institutes of Health, Bethesda, MD, USA) was used to quantify the relative protein levels. The expression of LC3B protein in the cells treated with rapamycin, 3-MA, puerarin + 3-MA, and transfected with miR-204 NC, miR-204 mimics and miR-204 inhibitor was also detected using this method.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Osteoblastic MC3T3-E1 cells were incubated with puerarin (1 μM) for 72 h, and total RNA was extracted using TRIzol reagent (Takara Bio, Inc.). The reverse-transcription reaction solution (20 μl) contained 10.0 μl 5X Reverse Transcription Mix (Takara Bio, Inc.), 1.0 μl Stem-loop RT primers (GenScript), 500 ng miRNA, 2.0 μl HiScript Enzyme Mix (cat. no. R223; Vazyme) and RNase-free water. The PCR amplification conditions consisted of 25˚C for 5 min, 45˚C for 50 min and 85˚C for 50 min. Specific miRNA stem-loop RT primers for mouse miR-204 and internal control U6 were designed and synthesized by Shanghai Sangon Pharmaceutical Co. Ltd. (Shanghai, China). Primers used in the stem-loop RT-qPCR were as follows: RT primer, 5'-GTCGTATCCAGTGCA GGGTCCGAGGTATTCGCACTGGATACGACAGGCAT-3'; PCR upstream primer, 5'-GCGGCGGTTCCCTTTGTCAT CC-3' and downstream primer 5'-ATCCAGTGCAGGGTC CGAGG-3' for miR-204; PCR upstream primer, 5'-CTCGCT TCGGCAGCACA-3' and downstream primer, 5'-AACGCT TCACGAATTTGCGT-3' for U6. The two-step PCR amplification conditions were: pre-denaturation of 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, 60˚C for 34 sec. The fluorescence signal detection was assessed using the Mx3000P Real-Time PCR system (Stratagene; Agilent Technologies, Inc.).

Bioinformatics predicts miR-204 and mouse LC3B gene binding sites. The binding sites of mouse LC3B and miR-204 were predicted using TargetScan (http://genes.mit.edu/targetscan).

Overexpression and inhibition of miR-204. Mouse miR-204 mimics, inhibitor and negative control were obtained from Shanghai Gene Pharma Co., Ltd. Cells were inoculated into 6-well plates at a density of $1x10^5$ per well and transfected using 4 μl Lipofectamine 2000 (Invitrogen; Semefield Technology) according to the manufacturers protocol when the cells reached 90% confluence. The control cells were transfected with only 4 μl transfection reagent. Then the cells were incubated in a humid 5% CO_2 incubator at 37˚C for 48 h. Cell preparation was used for RT-qPCR analysis of miR-204, transmission electron microscopy and western blot analysis of LC3B.

Statistical analysis. Data are expressed as mean ± standard deviation. Data were analyzed using one-way analysis of variance with SPSS software (version, 17.0; SPSS Inc.) or Student's t-tests. Bonferroni post hoc test was used to compare the differences between groups. $P<0.05$ is considered to indicate a statistically significant result.

Results

Puerarin promotes osteoblast viability, differentiation and mineralization. It was first ascertained whether puerarin affects the viability, differentiation and mineralization of osteoblasts. CCK-8 results showed that the viability of the osteoblastic MC3T3-E1 cells treated with different concentrations of puerarin (Pue) (0.1, 1 and 10 μM) for 24, 48 and 72 h was significantly higher than that noted in the control cells (except for 0.1 and 10 μM puerarin at 24 h) and puerarin at the concentration of 1 μM showed the highest effect at 72 h ($P<0.05$; Fig. 1A). The cells treated with 1 μM puerarin for 72 h also had a significantly higher ALP activity ($P<0.05$; Fig. 1B). In addition, the effect of puerarin on osteoblast mineralization was further tested by calculating the mineralized nodule area through Alizarin Red S staining. Compared with the control group, the formation of mineralized nodules was increased significantly in the cells treated with 1 μM puerarin for 7, 14 and 21 days ($P<0.05$; Fig. 1C and D).

Puerarin upregulates the expression of Beclin1 and LC3B and promotes the formation of autophagosome. The expression levels of Beclin1 and LC3B-II/LC3B-I were used to compare the intensity of autophagy. Puerarin at concentrations of 0.1 and 10 μM did not effectively promote osteoblast viability at 24 h; therefore, the expression levels of Beclin 1 and LC3B protein were observed at 48 and 72 h compared with levels at 0 h. The results ($P<0.05$; Fig. 2A and B) showed that after puerarin treatment for 48 and 72 h, Beclin1 and LC3B expression was significantly upregulated when compared with that at 0 h and puerarin showed the greatest effect at 72 h. The results suggested that the expression levels of Beclin1 and LC3B-II/LC3B-I proteins were upregulated in the puerarin-treated cells within 72 h and the effect was time-dependent. Therefore, in the following experiments 72 h for drug action time was selected. Next, we compared treatment of puerarin with 3-MA, rapamycin (Rap) and puerarin (Pue)+3-MA to confirm the intensity of puerarin in promoting autophagy. Compared with the blank control group, puerarin showed the highest promotive effect on LC3B expression, which exceeded that of pue+3-MA and rapamycin, while 3-MA significantly inhibited the expression of LC3B. For Beclin1 expression, rapamycin showed the highest upregulation effect better than that of puerarin. 3-MA exhibited an obvious inhibitory effect ($P<0.05$; Fig. 2C and D). The results showed that puerarin treatment significantly caused upregulation of autophagy-related proteins, especially significant promotion of the expression of LC3B. In addition, puerarin did not significantly upregulate Beclin1 expression after addition of 3-MA, but still upregulated LC3B expression. This suggests that the regulation of puerarin-induced autophagy may be related to the mediation of LC3B.

The ultrastructure of the cells were next observed by transmission electron microscopy, in order to confirm the occurrence of autophagy intuitively. The morphological distribution of organelles, nuclei and chromosomes was normal in the control and 3-MA-treated cells. There were many ring-shaped substances in the cytoplasm of the cells treated with puerarin, which had a double-layer membrane structure specific to autophagy (Fig. 2E). The results showed

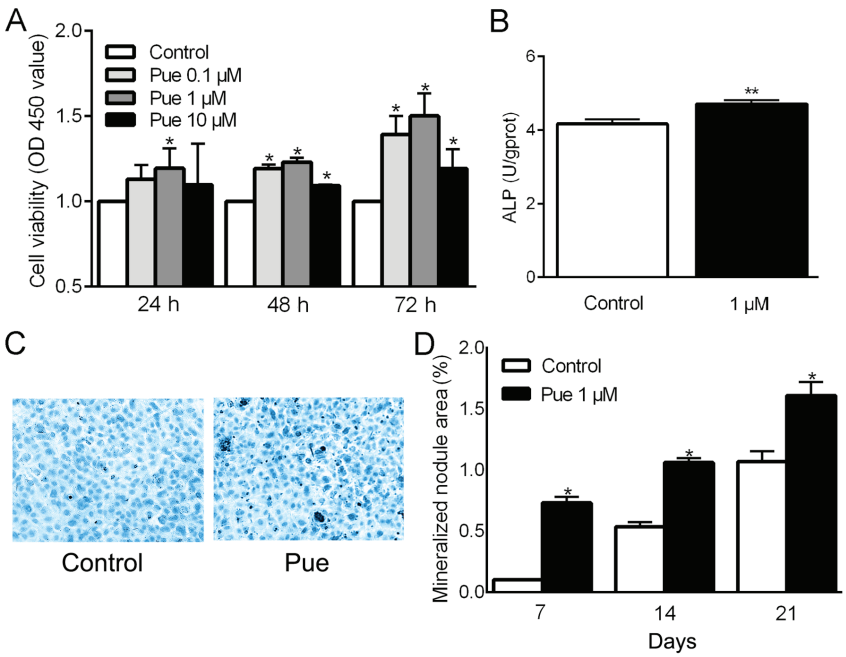

Figure 1. Effects of puerarin on MC3T3-E1 cell viability, differentiation and mineralization. (A) Effects of 0.1, 1 and 10 μM puerarin on osteoblast cell viability at 24, 48 and 72 h detected by CCK-8 assay, compared with a control treated with serum-free medium. (B) Effect of 1 μM puerarin on ALP activity in osteoblasts at 72 h. (C) Alizarin red staining for detection of mineralized nodules under optical microscope (magnification, x100). (D) Relative mineralized nodule area of each group was tested after treatment with 1 μM puerarin for 7, 14 and 21 days, respectively. *P<0.05 vs. the control. Pue, puerarin; ALP, alkaline phosphatase.

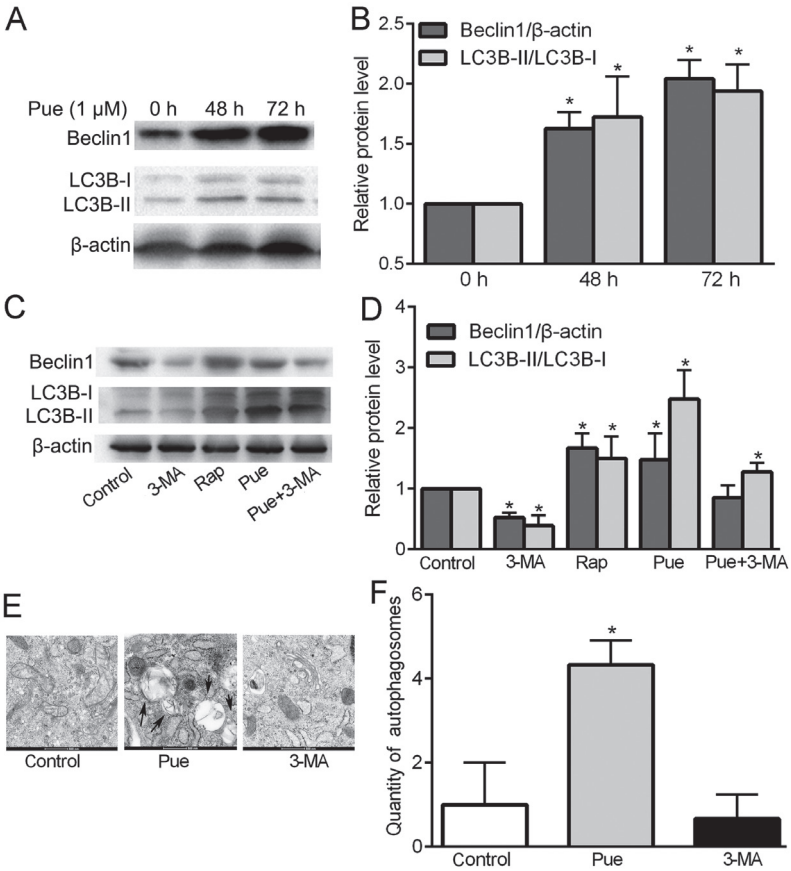

Figure 2. Effects of puerarin on autophagy. (A) Expression levels of Beclin1 and LC3B proteins were measured after osteoblastic MC3T3-E1 cells were treated with 1 μM puerarin for 48 and 72 h by western blotting, compared with a control (treated with puerarin for 0 h). (B) Quantification of A. (C) The protein levels of Beclin1 and LC3B were tested after cells were treated with puerarin (Pue), 3-MA (75 μM), rapamycin (rap) (100 μM) or Pue+3-MA for 72 h. (D) Quantification of C. (E) Representative images of autophagosomes (black arrows) were captured by transmission electron microscopy (scale bar, 500 nm). (F) Number of autophagosomes in the same field of vision, compared with a control. *P<0.05 vs. the control. LC3, microtubule-associated light chain 3; Pue, puerarin; 3-MA, 3-methyladenine; rap, rapamycin.

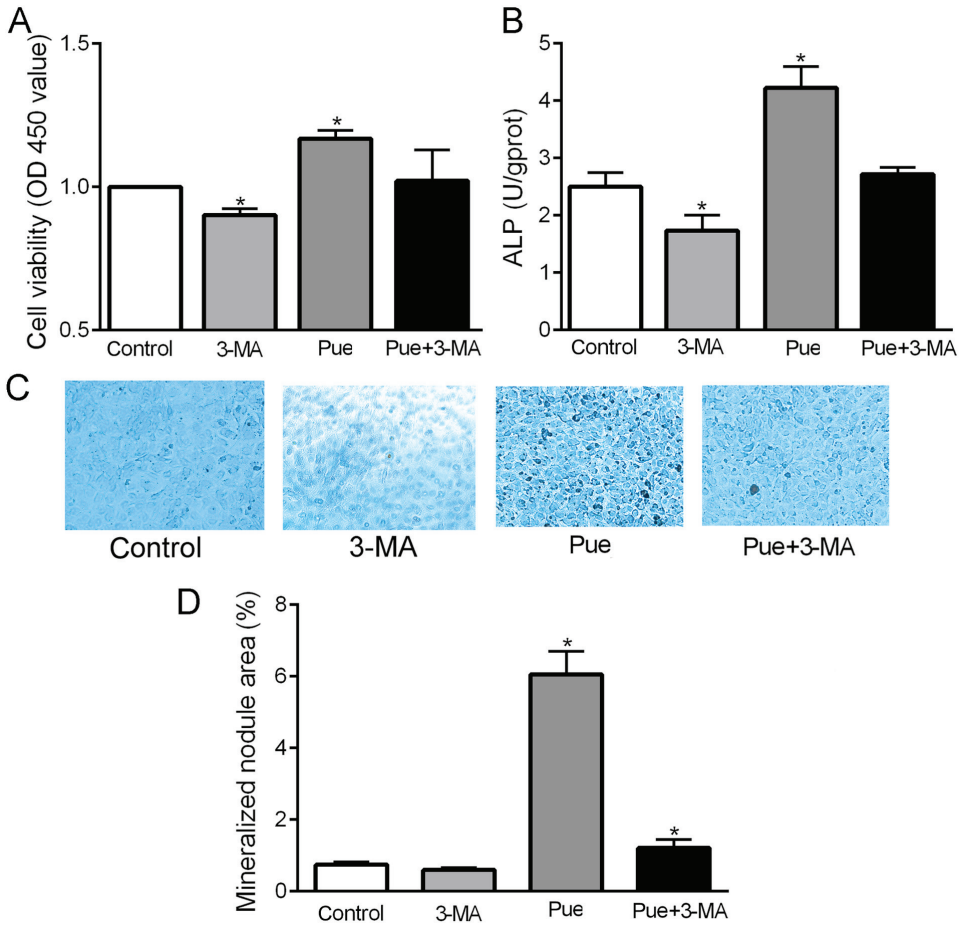

Figure 3. Effects of autophagy on osteoblastic MC3T3-E1 cell viability, differentiation and mineralization. (A) Cells were treated with puerarin (Pue) (1 μM), 3-MA (75 μM) or puerarin (Pue)+3-MA for 72 h. The cell viability was assessed by CCK-8 assay. (B) Alkaline phosphatase (ALP) activity was tested after cells were treatment with Pue (1 μM), 3-MA (75 μM) or Pue+3-MA for 72 h (C) Alizarin red staining was used to evaluate the mineralized nodules under an optical microscope (magnification, x100). (D) Relative mineralized nodule area of each group following treatment with Peu (1 μM), 3-MA (75 μM) or Peu+3-MA for 14 days. *P<0.05 vs. the control. 3-MA, 3-methyladenine.

that puerarin induced the formation of autophagosomes in the osteoblasts compared with that noted in the control and 3-MA-treated cells (P<0.05; Fig. 2F). This further confirmed that puerarin promotes autophagy of osteoblasts.

Inhibition of autophagy reduces the viability and differentiation of MC3T3-E1 osteoblasts, but has little effect on mineralization. The effects of autophagy on the viability, differentiation and mineralization of mouse MC3T3-E1 cells were observed. Compared with the blank control, the viability and ALP activity were significantly decreased following treatment with 3-MA (P<0.05; Fig. 3A and B), but the area of mineralized nodules exhibited no significant change (P<0.05; Fig. 3C and D). These results suggest that inhibition of autophagy can reduce the viability and differentiation of MC3T3-E1 osteoblasts, but has little effect on mineralization.

miR-204 regulates LC3B and affects autophagosome formation in MC3T3-E1 cells. miR-204 is a key regulator of bone growth, which participates in the viability and differentiation of osteoblasts. Our previous research demonstrated that low concentrations of puerarin promote osteoblast proliferation and differentiation by downregulating the expression of miR-204 by targeting Runx2 (14). In the present study, the effect of puerarin on miR-204 expression was examined by stem-loop RT-qPCR. The expression level of miR-204 was significantly decreased following culturing of the MC3T3-E1 cells with 1 μM puerarin for 72 h, compared with that of the cells in the absence of puerarin (Control) (P<0.05; Fig. 4A). The results demonstrated that puerarin downregulated the expression of miR-204. Combined with the previous validation that puerarin promotes osteoblast viability and differentiation through autophagy upregulation, the authors attempted to ascertain the relationship between miR-204 and autophagy. Bioinformatic analysis showed that the binding site of miR-204 in LC3B was a widely conserved element between 44 and 50 bp of the LC3B 3-'UTR, suggesting that LC3B may be a downstream target of miR-204 (Fig. 4B). Therefore, it was speculate that miR-204 can affect the level of autophagy by regulating LC3B. Furthermore, the effects of the overexpression and inhibition of miR-204 on LC3B were investigated. The results of RT-qPCR (P<0.05; Fig. 4C) showed that transfection with miR-204 mimics or miR-204 inhibitor significantly increased or decreased, respectively, the expression of miR-204 mRNA. In addition, the results of the western blotting (P<0.05; Fig. 4D and E) showed that overexpression of miR-204 significantly decreased the expression of LC3B protein, while inhibition of miR-204 had

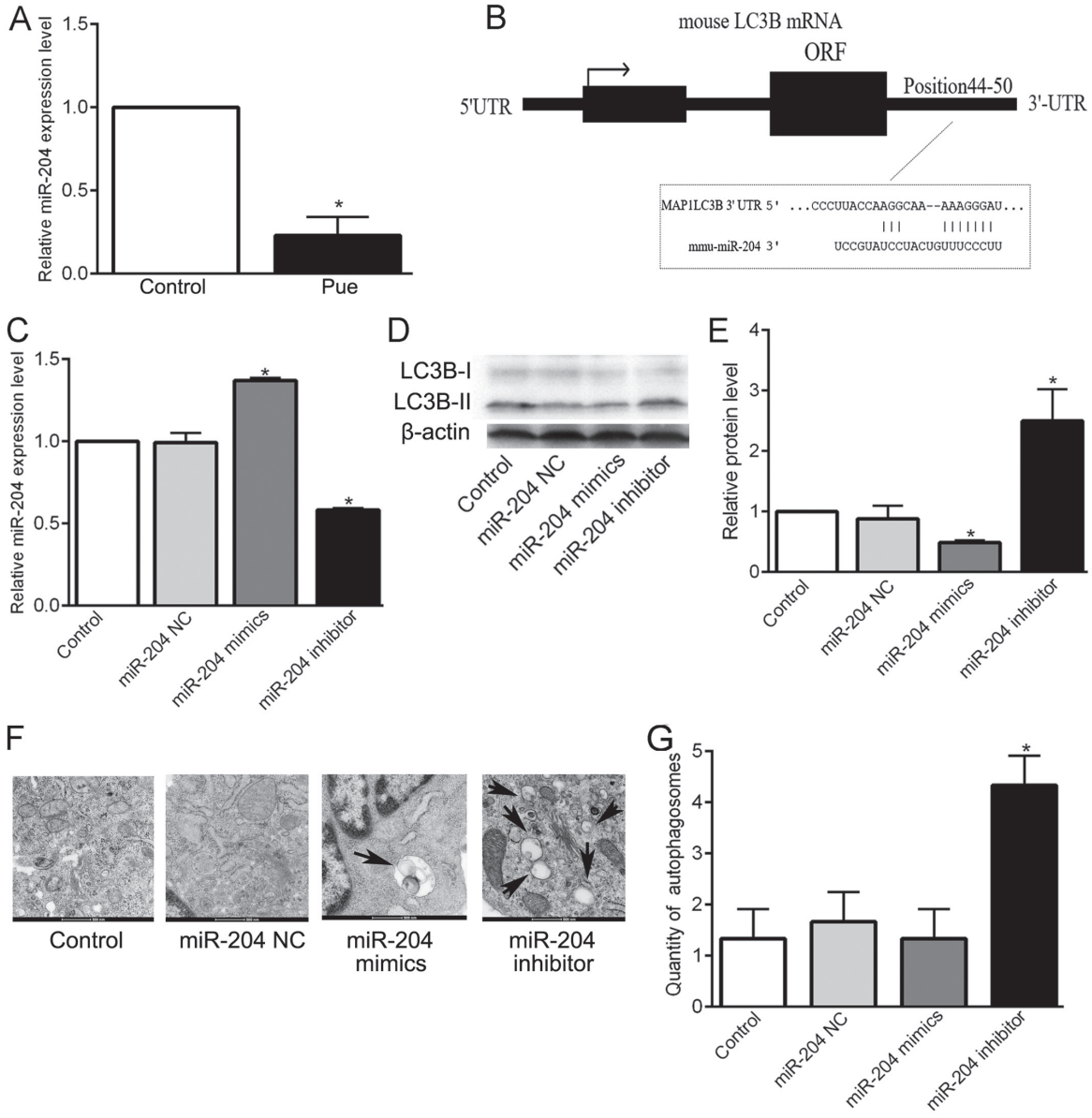

Figure 4. miR-204 regulates LC3B and autophagosome formation. (A) Osteoblastic MC3T3-E1 cells were treated with 1 μM puerarin for 72 h, and the miR-204 expression level was examined by stem-loop reverse transcription-quantitative polymerase chain reaction. (B) Schematic of the miR-204 target sites within the mouse LC3B WT 3'-UTR. (C) The expression level of miR-204 mRNA following transfection with miR-204 mimics, miR-204 inhibitor or miR-204 negative control (NC). (D) The expression of LC3B in osteoblasts at 72 h following transfection with miR-204 mimics, miR-204 inhibitor or miR-204 NC. (E) Quantification of D. (F) Representative images of autophagosomes (black arrows) were captured by transmission electron microscopy (scale bar, 500 nm). (G) Number of autophagosomes in the same field of vision, compared with the control. *P<0.05 vs. the control. miR, microRNA; LC3, microtubule-associated light chain 3; Pue, puerarin; UTR, untranslated region.

the opposite effect. The results showed that the expression of LC3B protein in osteoblasts is inversely regulated by miR-204. Furthermore, in a previous study, double luciferase reporter gene indicated the direct regulation of miR-204 on LC3B in renal clear cell carcinoma cells (15). Although this result was not validated in osteoblasts, it also supports our hypothesis to some extent. Then, electric mirror analysis showed that, compared with the blank control, there was no significant difference in the number of autophagosomes in the negative control and miR-204 mimic group, while the miR-204 inhibitor significantly increased the number of autophagosomes in the osteoblastic MC3T3-E1 cells (P<0.05; Fig. 4F and G). In conclusion, the results indicate that miR-204 regulates LC3B-mediated autophagy.

Discussion

The development of osteoblasts generally occurs in three stages: Proliferation, maturation of the extracellular matrix, and mineralization (16). At the maturation stage of the extracellular matrix, an increase in the synthesis and secretion of alkaline phosphatase (ALP), a homodimer glycoprotein secreted by osteoblasts, is considered the early stage of osteoblast differentiation and a prerequisite for the beginning of mineralization (17). Mineralization is the last stage of osteoblast differentiation and the primary marker of bone formation. Shortages or the inactivity of osteoblasts are two of the main pathological bases of osteoporosis. Promoting the proliferation, differentiation, and mineralization of osteoblasts

and improving their function are of great significance to preventing and treating osteoporosis.

During autophagy, cytoplasmic materials surrounded by bilateral membrane structures are called autophagosomes, the direct markers of autophagy. Autophagosome can bind with lysosomes to degrade related cytoplasmic structures. In addition, autophagy also occurs during starvation, quality control of intracellular protein organelles, inhibition of tumorigenesis, and antigen presentation. Ideally, evidence consistently shows that autophagy also plays an important role in cell differentiation and development. Beclin-1 and microtubule-associated light chain 3 (LC3) are the major autophagic proteins involved in the formation of autophagosomes (18,19). Early changes in these proteins may affect our further study of bone development. Over the past few years, more and more attention has been paid to the effect of autophagy on osteoporosis. The effects of autophagy on osteoblasts are also controversial. Some studies have found that autophagy promotes osteoblast proliferation and differentiation but does not promote mineralization. This effect may be related to the upregulation of BMP2, phosphorylation of SMAD1/5/8 proteins, transcription of RUNX-2, OSX and SMAD-7 expression and activation of the MEK/ERK pathway (20-22). However research has also shown that autophagy can promote mineralization (23). Our studies have found that puerarin, at a concentration of 1 μM, promotes the viability, differentiation and mineralization of osteoblasts. It also increased the expression of autophagy-related factors LC3B and Beclin 1, as well as the formation of autophagosomes. Then inhibition of autophagy significantly reduced the viability and differentiation of osteoblasts, but had no significant effect on mineralization. Therefore, we speculated that the change in the level of autophagy may not be the main reason why puerarin promotes the mineralization of osteoblasts. We then investigated the mechanism through which autophagy promotes osteoblast proliferation and differentiation. The results showed that miR-204/LC3B participated in the regulatory mechanism. miRNAs are a large family of small non-coding RNAs that regulate gene expression. miR-204 is a negative regulator involved in the regulation of a variety of biological activities (24). Research indicates that it is downregulated during puerarin-promoted osteoblast viability and differentiation (14). In the present study, puerarin downregulated the expression of miR-204, and bioinformatics analysis revealed that the binding site for miR-204 in LC3B mRNA is a widely conserved element located between 44 and 50 bp of the 3'-UTR of LC3B. Furthermore, overexpression of miR-204 significantly reduced the expression of LC3B protein and the number of autophagosomes, whereas inhibition of miR-204 was reversed. The above results confirmed that puerarin promotes the viability and differentiation of MC3T3-E1 cells by enhancing LC3B-mediated autophagy by downregulating miR-204, but there is still a lack of evidence confirming the direct effect of miR-204 on LC3B at the gene level. Thus, the specific mechanism requires further study. In the present study, changes in LC3B regulated by miR-204 were observed and assessed. However, autophagy-related regulator Beclin1 was also upregulated after puerarin treatment. Based on software prediction and bioinformatic analysis, miR-204 cannot directly target Beclin1. Nevertheless, it has been found that microRNAs usually play a role in clusters (25). The effect of other microRNAs in the expression profile of Beclin1 after puerarin treatment still remains unclear. In fact, studies have shown that autophagy-related factors, such as Beclin1, can directly or indirectly regulate the expression of microRNAs (26,27). This suggests that regulatory factors and microRNAs are not independent, and they regulate each other in a bidirectional manner. In addition, the downstream molecule of autophagy has not been clearly studied, although it may be related to the mTOR pathway or osteogenesis-related markers such as Runx2 and related animal experiments can further explain the issue. In summary, the results from the present study demonstrated that puerarin significantly promoted the viability, differentiation and mineralization of osteoblasts. It also increased the expression of autophagy-related factors such as LC3B and Beclin1, as well as the formation of autophagosomes. However, the cell viability and differentiation decreased after inhibition of autophagy. Furthermore, we found that downregulation of miR-204 promoted protein expression of autophagy-associated factor LC3B and formation of autophagosomes. The above results suggest that puerarin promotes the viability and differentiation of MC3T3-E1 cells by enhancing LC3B-mediated autophagy by downregulating miR-204. These findings provide a better understanding of the role of puerarin in bone biology.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QF, SYC, RY, XWZ and XQZ contributed to the study design, statistical analyses, data interpretation, manuscript preparation and the literature search. FMZ contributed to the study design, data collection and statistical analyses. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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