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RESEARCH ARTICLE

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Puerarin inhibits TRPM3/miR-204 to promote MC3T3-E1 cells proliferation, differentiation and mineralization

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Puerarin is an isoflavonoid phytoestrogen extracted from the root of Radix Pueraria, has attracted increasing attention because of its beneficial effects on anti-osteoporosis, but the molecular mechanisms underlying its actions on osteoblasts are not fully understood. The current study aimed to investigate the effect of puerarin on MC3T3-E1 osteoblastic cells proliferation, differentiation and mineralization, in vitro and its underlying mechanisms. The results indicated that puerarin significantly promoted the osteoblasts proliferation, enhanced alkaline phosphatase activity and increased the formation of mineralized nodules. Following treatment with puerarin, the expression levels of transient receptor potential Melastatin 3 (TRPM3) and microRNA-204 (miR-204) were decreased, whereas that of Runt-related transcription Factor 2 (Runx2) increased. TRPM3-small interfering RNA and 2-aminoethoxydiphenyl borate (2-APB, inhibitor of TRPM3) promoted the expression of Runx2 and thus improved the development of osteoblasts, but pregnenolone sulfate, which is the agonist of TRPM3, inhibited the effects. In addition, puerarin induced the changes of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and extracellular Ca^{2+} concentration ($[Ca^{2+}]_0$) through TRPM3 might be involved in the biological process of MC3T3-E1 cells. These results suggested that puerarin may promote MC3T3-E1 cell proliferation, differentiation and mineralization, which may be related to the downregulation of TRPM3/miR-204 and following regulating $[Ca^{2+}]_i$ and $[Ca^{2+}]_0$, and activation of Runx2.

KEYWORDS

MC3TC-E1 cells, microRNA-204, proliferation, Puerarin, Runx2, TRPM3

1 | INTRODUCTION

Osteoporosis is a common and frequently occurring disease that seriously threatens the health of the elderly, it has become a worldwide problem that increases health spending and the financial burden. It is generally believed that osteoblasts are the most important material basis for bone formation; their defects in proliferation and differentiation are considered to be one of the fundamental causes of osteoporosis.

Puerarin, an isoflavonoid phytoestrogen extracted from the root of Radix Pueraria, is widely prescribed for patients in China for various medicinal purposes. It has been used for treatment of angina pectoris and hypertension in China for several decades (Wang et al., 2003; Wang, Wu, Chiba, Yamada, & Ishimi, 2005). In vivo experimental studies reported that puerarin could reduce bone reabsorption, increase bone mineral density, promote bone formation, and has fewer side effects compared to estrogen (Li et al., 2016; Li, Liu, & Jia, 2014). Our previous research has suggested that puerarin may promote the proliferation of mouse MC3T3-E1 osteogenic cells by regulating the expression of Runx2-targeting miRNAs in vitro (Zhang, Zhou, Zeng, Zhao, & Zhan, 2016). Recently, puerarin has received extensive attention because of its possible role in the prevention of osteoporosis. However, the molecular mechanism of puerarin against osteoporosis remains to be further elucidated.

Runt-related transcription Factor 2 (Runx2) is a key bone growth regulatory factor in osteoblasts, which has been thought to be involved in the proliferation and differentiation (Liu et al., 2014). MiR-204 is a small single-stranded noncoding RNA molecule and function as a regulator of gene expression. According to the bioinformatics analysis, miR-204 is located in the sixth intron of transient receptor potential Melastatin 3 (TRPM3) gene, the expression of miR-204 was linearly correlated with that of TRPM3 gene in vivo (Ding et al., 2015) and

in vitro (Ying et al., 2013) strongly indicating that TRPM3 and miR-204 share common regulatory mechanisms. TRPM3 gene is a member of the M subfamily of the transient receptor potential (TRP) family, encodes proteins that from transient receptor potential nonselective cation channel on the plasma membrance, which is permeable to Ca^{2+} ions. Ca^{2+} is an intracellular messenger responsible for controlling cellular processes, including gene expression, cell cycle, and cell death. The changes of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and extracellular Ca^{2+} concentration ($[Ca^{2+}]_O$) have important regulatory effects on osteoblast development (Hu et al., 2014; Wang et al., 2014; Zayzafoon, 2006). Currently, very little is known about possible regulatory mechanisms of TRPM3 on osteoblast, the regulation of TRPM3 expression by puerarin also needs to be studied.

In the present study, we examined the promoting effects of puerarin on MC3T3-E1 osteoblastic cells proliferation, differentiation and mineralization, and focused on the regulatory mechanism involving TRPM3/miR-204 and Runx2. We also identified that puerarin induced the changes of $[Ca^{2+}]_i$ and $[Ca^{2+}]_O$ through TRPM3 might be involved in the promoting effects in MC3T3-E1 cells.

2 | MATERIALS AND METHODS

2.1 | Reagents

Puerarin was purchased from the National Institutes for Food and Drug Control (Beijing, China). From Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), 17β-estradiol (E2) was brought. α-modified eagle's medium (α-MEM) and penicillin–streptomycin solution were purchased from Gibco Laboratories (Grand Island, NY, USA). Fetal bovine serum was purchased from ScienCell Research Laboratories (San Diego, CA, USA). Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kamimashiki-gun, Kumamoto, Japan). Alkaline phosphatase (ALP) kit and calcium detection kit (C004-2) were provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Fura-3/AM was a product of Beyotime (Shanghai,China). Alizarin red S and 2-aminoethoxydiphenyl borate (2-APB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Pregnenolone sulfate (PS) was purchased from Selleckchem (Houston, TX, USA).

2.2 | Osteoblasts culture

The mouse preosteoblastic MC3T3-E1 cells was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in α-modified eagle's medium supplemented with 10% Fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin, maintained at 37 °C in a humidified 5% CO_2 incubator (SANYO, Japan). Cells were washed with PBS and collected using 0.25% trypsin-EDTA for 1 min when the cells reached 80% confluence.

2.3 | Determination of cell proliferation (CCK-8 method)

Cells were seeded at 1×10^4 per well in 96-well culture plate. The cell culture medium was discarded after 24 hr, and different treatment factors applied to each group. After 24, 36, or 48 hr, 10 ul of CCK-8 solution was added to each well and incubated at 37 °C for 1 hr. The cell viability was assessed by measuring the absorbance at 450 nm using Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

2.4 | Determination of activity expression of ALP

Cells were cultured in a 24-well culture plate at a density of 5×10^4 per well. After treated with puerarin for 48 hr, cells were washed 3 times with PBS. Then, the absorbance values were measured with ALP kit according to the manufacturer's instruction. The ALP activities were aslo examined after cells transfected with TRPM3-small interfering RNA or negative control.

2.5 | Count of mineralized nodule

In each well, 5×10^4 cells were added to 24-well culture plate. After 24 hr, the cells were treated with puerarin, PS, or 2-APB. 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 Days 14, 21, and 28, respectively. Five fields were randomly selected for each well under low magnification, and the relative mineralized nodule areas were analyzed by Image J software (Rawak Software Inc., Stuttgart, Germany).

2.6 | Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurement

Cells were seeded at 1×10^5 per well in six-well culture plate. After treated with puerarin for 2, 24, or 48 hr, cells were collected and resuspended in 1 ml PBS. Flou-3/AM (final concentration, 2.5 μmol/L) was added and incubated for 45 min, protected from light in a 37 °C carbon dioxide incubator. The average fluorescence intensity of cells in each group was measured by flow cytometry (Beckman Coulter, USA) to reflect the intracellular calcium concentration. The excitation wavelength was 488 nm, and the emission wavelength was 530 nm.

2.7 | Extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) measurement

Cells were seeded in six-well culture plate at 1×10^5 per well. The cell culture medium was discarded after 24 hr, and different treatment factors applied to each group. After treated with puerarin for 2, 24, or 48 hr, the cell culture supernatants were collected for the detection of extracellular calcium concentration according to the manufacturer's instructions.

2.8 | RNA transfection

When the cell density reached 60% confluence, small interfering RNA duplexes specific for mouse TRPM3 or negative control were transfected using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA). The mouse miR-204 mimics, miR-204 inhibitor and negative control were transfected using Lipofectamine 2000 according to the manufacturer's instructions. The RNAs were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China).

2.9 | Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from MC3T3-E1 cells using the Trizol method (Invitrogen, USA); purity and concentration of the isolated RNA were detected with protein nucleic acid analyzer (BioDrop μLite; Biodrop, Cambridge, UK). The RNA was then transcribed to complementary DNA with reverse transcription kit (Takara Bio, Inc., Otsu, Japan), following the manufacturer's protocol. Complementary DNAs were amplified by polymerase chain reaction (PCR) with the following primers: TRPM3 (5′-CTTTCGGACCCTCTACCACA-3′ and 5′-CACC TCTTCCTCACGCTTCT-3′), Runx2 (5′-CGGACGAGGCAAGAGT TTCA-3′ and 5′-GGATGAGGAATGCGCCCTAA-3′), and β-actin (5′-GTGCTATGTTGCTCTAGACTTCG-3′ and 5′-ATGCCACAGGATTCCA TACC-3′). PCR was performed under the following conditions: predenaturation of 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 34 s. The fluorescence signal detection was measured by Mx3000P real-time PCR system (Agilent Technologies Stratagene, USA). The miR-204 expression was examined by Stem-Loop RT-qPCR. Primers are the following: RT primer,5′-GTCG TATCCAGTGCAGGG TCCGAGGTATTCGCACTGGATACGACAGGCAT-3′; PCR upstream primer, 5′-GCGGCGGTTCCCTTTGTCATCC-3′; downstream primer 5′-ATCCAGTGCAGGGT-CCGAGG-3′ for miR-204; PCR upstream primer, 5′-CTCGCTTCGGCAGCACA-3′; downstream primer, 5′-AACGCTTCACGAATTTGCGT-3′ for U6. 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).

2.10 | Western blot

Protein was extracted from the cells with 200 μl RIPA buffer, including proteinase inhibitor cocktail (Santa Cruz Biotechnology, USA), and then the protein content was measured by bicinchoninic acid assay (BCA) method (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), according to the manufacturer's instructions. Protein was subjected to SDS-PAGE gels and electro-transferred onto polyvinylidene difluoride (PDVF) membrane. After blocking with 5% skimmed milk in TBS containing 0.1% Tween-20 (TBST) at room temperature for 1 hr, the protein samples were incubated with primary antibody (CST, USA) for TRPM3 (1:1000), Runx2 (1:2000), and β-actin (1:2000) in 5% skimmed milk at 4 °C overnight. After washed 3 times with TBST, the samples were incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:2000, CST, USA) in 5% skimmed milk for 1 hr. Following washed 3 times with TBST, the protein-antibody complexes were visualized by the enhanced chemiluminescent (ECL) detection system (Bio-Rad, USA). Intensity of the bands was measured with the Image Lab software assess relative protein levels.

2.11 | Statistical analysis

Results were expressed as mean ± standard deviation. Data comparisons were analyzed by Student's *t*-test or one-way ANOVA followed by Bonferroni's post hoc test. A value of $p < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Puerarin promotes MC3T3-E1 cell proliferation, differentiation and mineralization

The effect of puerarin on mouse osteoblastic cell line MC3T3-E1 cells viability was examined by exposing the cells to puerarin at various concentrations for 24, 48, and 72 hr. As shown in Figure 1a, the cells treated with puerarin had significantly higher viability compared with that of the control cells ($p < .05$), except those treated with puerarin at 10 μM concentration for 24 hr. The cells that were treated with 0.1 μM of puerarin had the highest viability among the three concentrations (the effect of positive Control E2 is not included), suggesting that a lower concentration of puerarin might be have a better promoting effect. After cultured in the presence of 0.1 μM puerarin for 48 hr, the cells had significantly higher ALP activity than the control ($p < .05$;

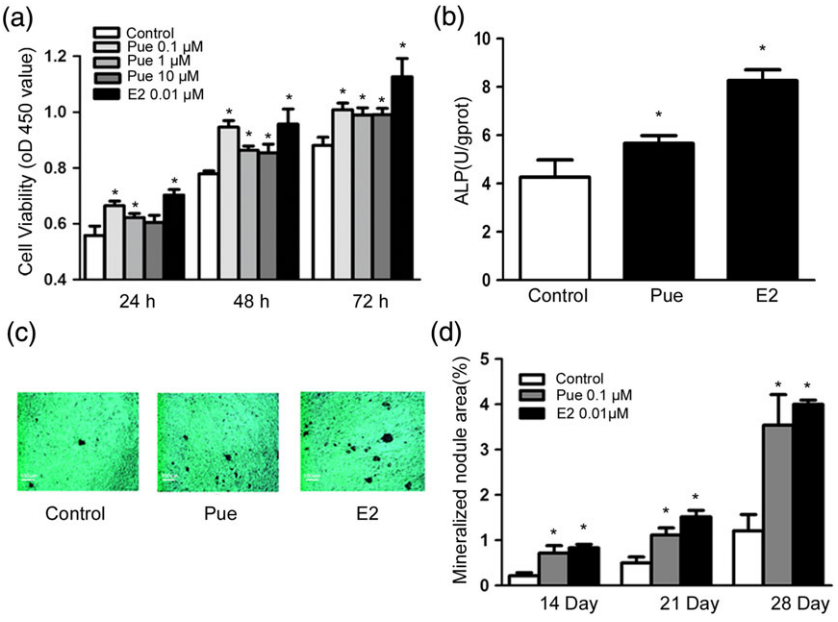

FIGURE 1 Effect of puerarin on MC3T3-E1 cell proliferation, differentiation and mineralization. (a) Cells were treated with puerarin at various concentrations (0.1, 1, 10 μM) for 24, 48, and 72 hr. The cell viability was assessed by CCK-8 assay. (b) Alkaline phosphatase (ALP) activity of the cells was tested after treatment with 0.1 μM puerarin for 48 hr. (c) Alizarin red staining for mineralized nodule under optical microscope (×100). (d) Relative mineralized nodule area of each group was tested after treatment with 0.1 μM puerarin for 14, 21, and 28 days, respectively. *$p < .05$, compared with the control [Colour figure can be viewed at wileyonlinelibrary.com]

Figure 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 puerarin for 14, 21, and 28 days ($p < .05$; Figure 1c,d).

3.2 | Puerarin downregulates the expression of TRPM3/miR-204

To evaluate the underlying mechanisms of puerarin mediated osteoblasts promoting effect, we examined the effect of puerarin on TRPM3 and miR-204 expression. Cells were treated with 0.1 μM puerarin for 48 hr, the expression of TRPM3 mRNA (Figure 2a) and protein (Figure 2b,c) was downregulated. Then, stem-loop RT-qPCR was used to detect the effect of puerarin on miR-204 expression. As shown in Figure 2d, the expression level of miR-204 was significantly decreased following culturing the cells with 0.1 μM puerarin for 48 hr. The results suggested that puerarin may downregulate the expression of TRPM3/miR-204.

3.3 | TRPM3/miR-204 inhibits the expression of Runx2

Runx2 is considered to be a master bone growth regulatory factor, involved in the proliferation and differentiation of osteoblasts. We next investigate whether TRPM3 and miR-204 play a role in the expression of Runx2. We first silenced TRPM3 gene with small interfering RNA(siRNA) and assessed Runx2 expression. Upon knockdown with specific siRNA for TRPM3, the mRNA level of TRPM3 was suppressed (Figure 3a), whereas the mRNA expression level (Figure 3b) and the protein expression level (Figure 3c,d) of Runx2 was increased. Then, cells were exposed to PS and 2-APB, which are agonist and inhibitor of TRPM3, respectively (Son, Yang, Park, Chang, & Shin, 2015). As shown in Figure 3e–g, mRNA and protein levels of Run2 were significantly decreased after the treatment of cells with 100 μM PS, while 75 μM 2-APB enhanced the expression levels. Next, the effect of miR-204 on Runx2 protein expression was investigated in MC3T3-E1 cells at 48 hr following transfection with miR-204 mimics, inhibitor or miR negative control. Western blotting analysis (Figure 3h,i) indicated that overexpression of miR-204 decreased the protein expression level of Runx2, whereas inhibition of miR-204 had the opposite effect.

3.4 | TRPM3/miR-204 inhibits MC3T3-E1 cell proliferation, differentiation and mineralization

To determine whether TRPM3 and miR-204 play a role in the proliferation, differentiation and mineralization in MC3T3-E1 cells, we first examined the effect of TRPM3 on cell proliferation. As shown in Figure 4a, the cells treated with 100 μM PS had lower viability compared with that of the control cells, whereas cells treated with 75 μM 2-APB had higher viability. Transfected with TRPM3 siRNA and the use of 2-APB increased the expression of ALP, whereas PS inhibited the expression of ALP after cultured for 48 hr (Figure 4b,c). The effect of TRPM3 on osteoblast mineralization was further investigated by examining mineralized nodule area. Compared with the control group, the formation of mineralized nodules was increased in the cells treated with 2-APB for 14 days, whereas that in PS group decreased (Figure 4d,e). In addition, miR-204 also reduced the cell activity (Figure 4f) and inhibited the expression of ALP (Figure 4g).

3.5 | Ca^{2+} influx regulated by TRPM3 is involved in the proliferation, differentiation and mineralization in MC3T3-E1 cells

In order to detect whether Ca^{2+} influx plays a role in the promoting effects of puerarin on MC3T3-E1 cells, we first explored the effects of $[Ca^{2+}]_0$ on the cells. As shown in Figure 5a,b, $[Ca^{2+}]_0$ could promote MC3T3-E1 cells proliferation and differentiation following treatment for 24, 36, and 48 hr. Then, the effect of TRPM3 on Ca^{2+} concentration was observed. TRPM3 silencing and inhibitor 2-APB increased $[Ca^{2+}]_0$, which may be caused by decreasing Ca^{2+} influx, whereas PS

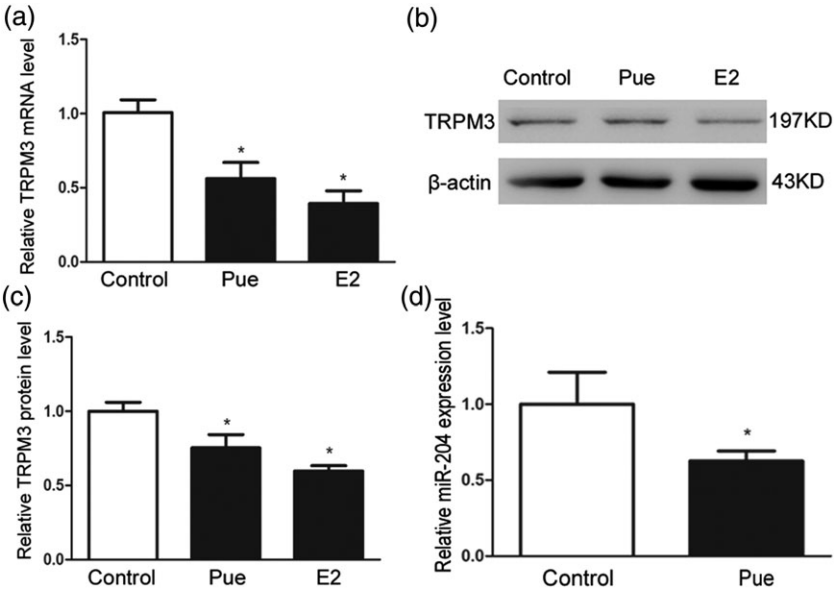

FIGURE 2 Effect of puerarin on transient receptor potential Melastatin 3 (TRPM3)/miR-204 expression in MC3T3-E1 cells. (a) TRPM3 mRNA expression level was examined after cells were treated with 0.1 μM puerarin for 48 hr. (b,c) The protein expression level of TRPM3 was measured after cells were treated with 0.1 μM puerarin for 48 hr. (d) Cells were treated with 0.1 μM puerarin for 48 hr, miR-204 expression level was examined by stem-loop reverse transcription-quantitative polymerase chain reaction. *$p < .05$, compared with the control

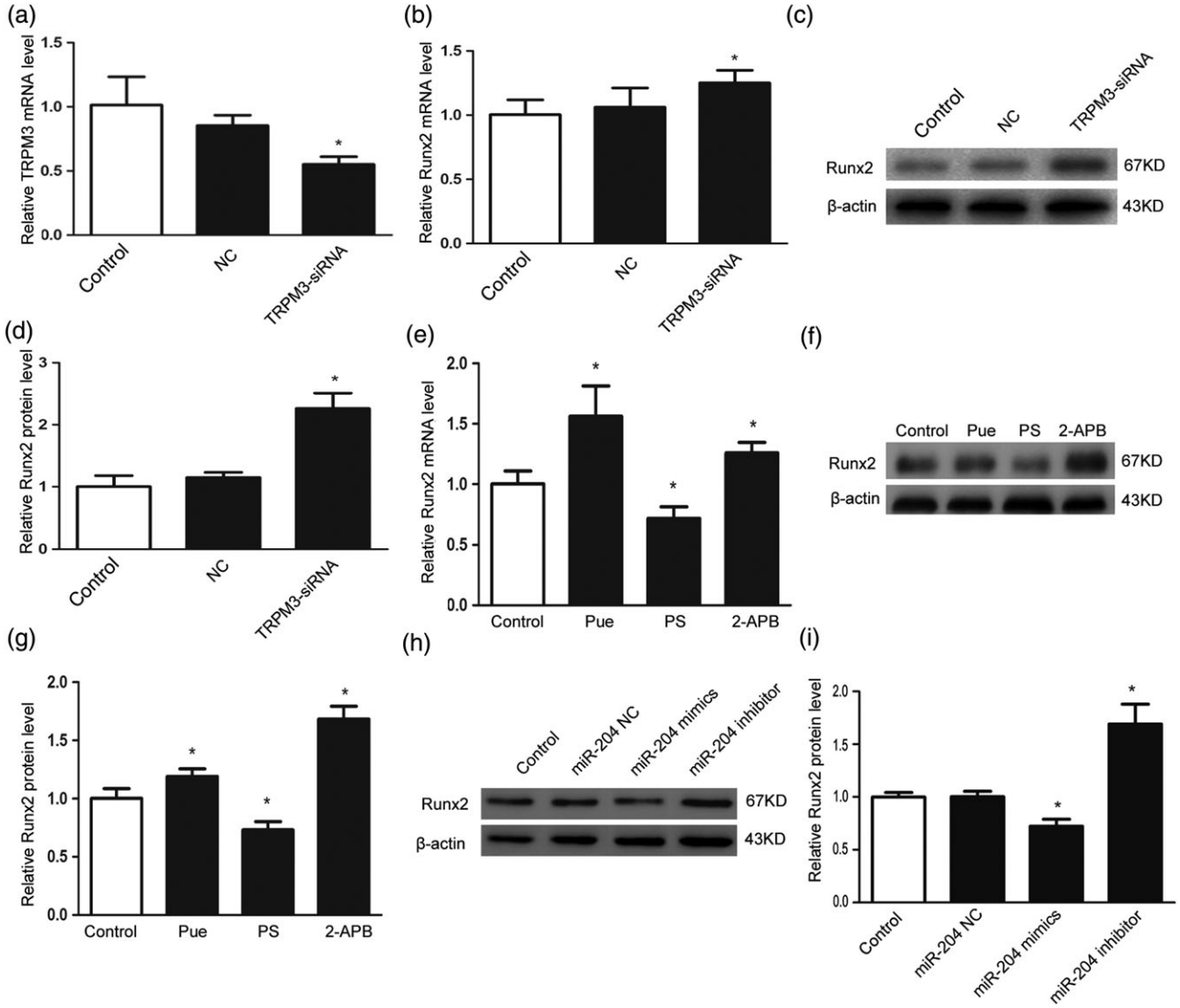

FIGURE 3 Effect of TRPM3/miR-204 on Runx2 expression in MC3T3-E1 cells. (a) Inhibition of transient receptor potential Melastatin 3 (TRPM3) with siRNA. The decreased mRNA expression of TRPM3 was measured by RT-PCR. (b) Runx2 mRNA expression level was measured after cells were transfected with TRPM3-siRNA for 48 h. (c,d) The protein expression level of Runx2 was examined following transfected with TRPM3-siRNA for 48 hr. (e) Cells were treated with PS (100 μM) or 2-APB (75 μM) for 48 hr, the mRNA level of Runx2 was determined by RT-PCR. (f,g) The protein level of Runx2 was tested after cells treated with PS (100 μM) or 2-aminoethoxydiphenyl borate (2-APB; 75 μM) for 48 hr. (h,i) Runx2 protein expression level was assessed following transfection with miR-204 mimics, inhibitor or miR negative control for 48 hr.* $p < .05$, compared with control

(agonist of TRPM3) had the opposite effect after cultured for 48 hr (Figure 5c,d). We next investigated the effect of puerarin on the $[Ca^{2+}]_i$ and $[Ca^{2+}]_O$ in MC3T3-E1 cells. Puerarin enhanced the increases in $[Ca^{2+}]_i$ and decreased $[Ca^{2+}]_O$ in a short time (2 hr), but reversed the effect after 48 hr (Figure 5e,f). In addition, the addition of 5 μM Ca^{2+} to the culture medium for 48 hr did not alter the effect of puerarin, PS, and 2-APB on cells proliferation (Figure 5g), indicating that the change of $[Ca^{2+}]_i$ is not the main cause of puerarin for promoting osteoblasts proliferation at 48 hr, the increased of Runx2 expression regulated by TRPM3/miR-204 may be the dominant factor. We speculate that the increase of $[Ca^{2+}]_i$ after a short time (2 hr) action of puerarin is for promoting proliferation and differentiation of the cells, the decrease of $[Ca^{2+}]_i$ in the later stage (48 hr) is due to the increase of $[Ca^{2+}]_O$ by the entry of Ca^{2+} into the extracellular space, which may be prepare for mineralization by combining with the extracellular matrix protein. These results suggested that Ca^{2+} might be involved in MC3T3-E1 cells proliferation, differentiation and mineralization at different time points.

4 | DISCUSSION

In general, the development of osteoblasts has been subdivided into three stages: proliferation, extracellular matrix maturation, and mineralization (Addison, Azari, Sorensen, Kaartinen, & McKee, 2007). ALP is a homodimeric glycoprotein secreted by osteoblasts, the increase of ALP synthesis and secretion during extracellular matrix maturation phase is considered to be an early event of osteoblast differentiation and a prerequisite for the beginning of mineralization (Hoac, Kiffer-Moreira, Millan, & McKee, 2013). Mineralization is the final stage of osteoblast differentiation and is an early sign of bone formation. The deficiency of osteoblast numbers or dysfunction of activity is one of

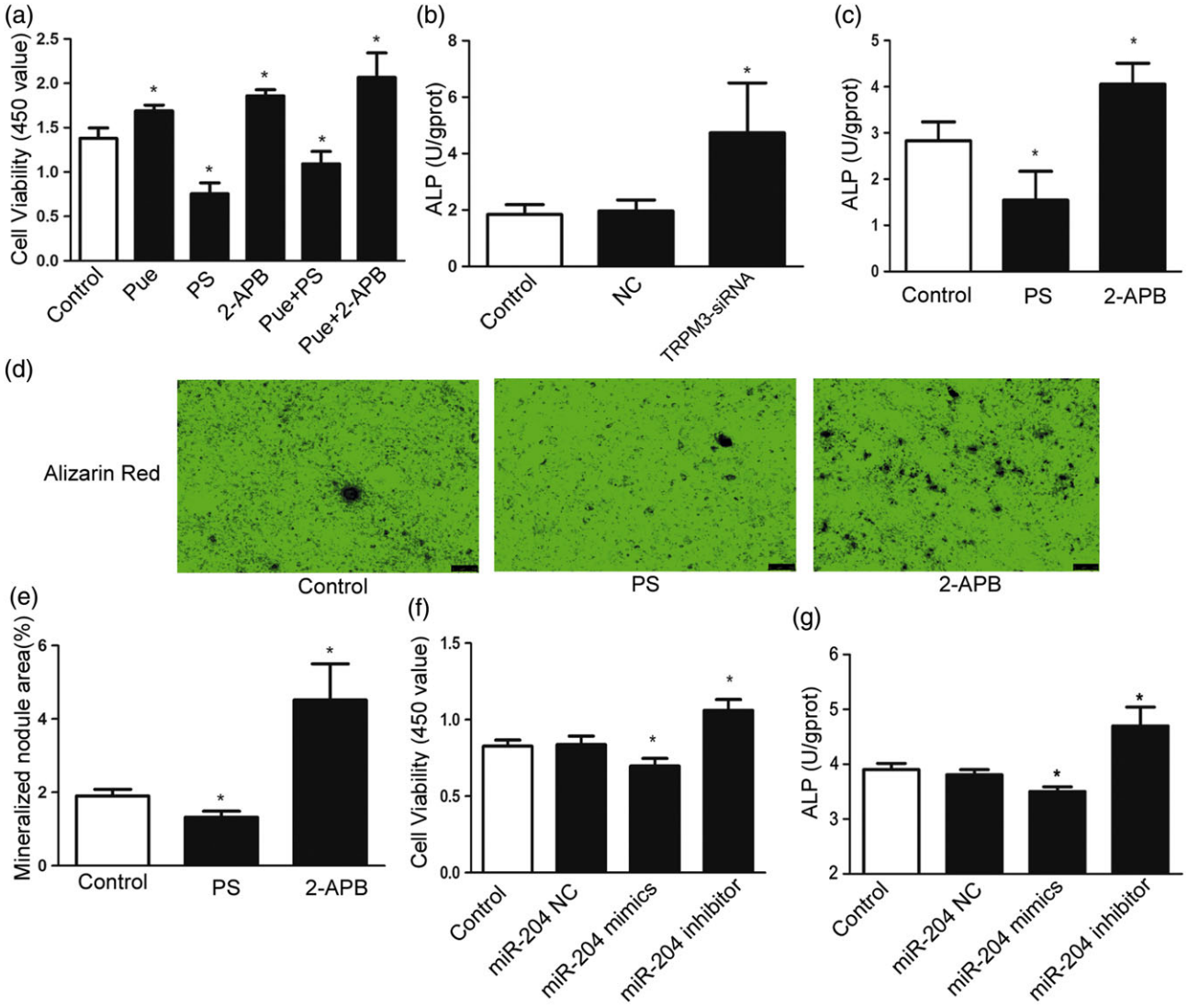

FIGURE 4 Effect of TRPM3/miR-204 on proliferation, differentiation and mineralization in MC3T3-E1 cells. (a) Cells were treated with puerarin (0.1 μM), pregnenolone sulfate (PS; 100 μM), or 2-aminoethoxydiphenyl borate (2-APB; 75 μM) for 48 hr. The cell viability was assessed by CCK-8 assay. (b,c) Alkaline phosphatase (ALP) activity was tested after cells were transfected with TRPM3-siRNA or treatment with PS (100 μM), 2-APB (75 μM) for 48 hr. (d) Alizarin red staining for mineralized nodule under optical microscope (×100). (e) Relative mineralized nodule area of each group treated with PS (100 μM) or 2-APB (75 μM) for 14 days. (f) Cell viability was detected by CCK-8 assay following transfection with miR-204 mimics, inhibitor or miR negative control for 48 hr. (g) ALP expression was examined following transfection with miR-204 mimics, inhibitor or miR negative control for 48 hr. *$p < .05$, compared with control [Colour figure can be viewed at wileyonlinelibrary.com]

the main pathological basis of osteoporosis, to promote osteoblast proliferation, differentiation and mineralization, and to improve the function of osteoblast, which may be particularly important for the prevention and treatment of osteoporosis.

MC3T3-E1 is an osteoblast precursor cell line derived from mouse calvaria and undergoes a temporal pattern of osteoblast development similar to bone formation in vivo, including ALP activity, extracellular matrix maturation, and mineralization. Thus, this cell has become a valuable cell model for studying osteoporosis in vitro (Lee & Choi, 2011; Yang et al., 2015). Runx2 is a key transcription factor involved in osteoblast proliferation and differentiation. It has been demonstrated that puerarin could increase the expression of Runx2 to promote osteoblast proliferation and differentiation (Sheu et al., 2012), which is consistent with our findings. Studies have reported that miR-204 inhibited osteogenesis of bone marrow mesenchymal stem cells and human aortic valve interstitial cells by negatively regulating the expression of Runx2 (Huang, Zhao, Xing, & Chen, 2010; Wang et al., 2015). However, little attention is paid to the effect of miR-204 on osteoblast proliferation and differentiation in vitro. The current study found that miR-204 and its host gene TRPM3 negatively regulated Runx2 expression in MC3T3-E1 osteoblasts. On the one hand, as previously study reported (Ying et al., 2013), the results may be suggested that the expression of miR-204 linearly correlated with that of TRPM3 gene in vitro. On the other hand, it may explain the possible molecular mechanism of puerarin-induced promoting effects on osteoblasts. Our results showed that puerarin reduced the expression of TRPM3/miR-204 to promote the expression of Runx2, which induced the proliferation and differentiation of MC3T3-E1 cell, but the regulation of puerarin on $[Ca^{2+}]_i$ and $[Ca^{2+}]_O$ may explain its molecular mechanism of promoting mineralization.

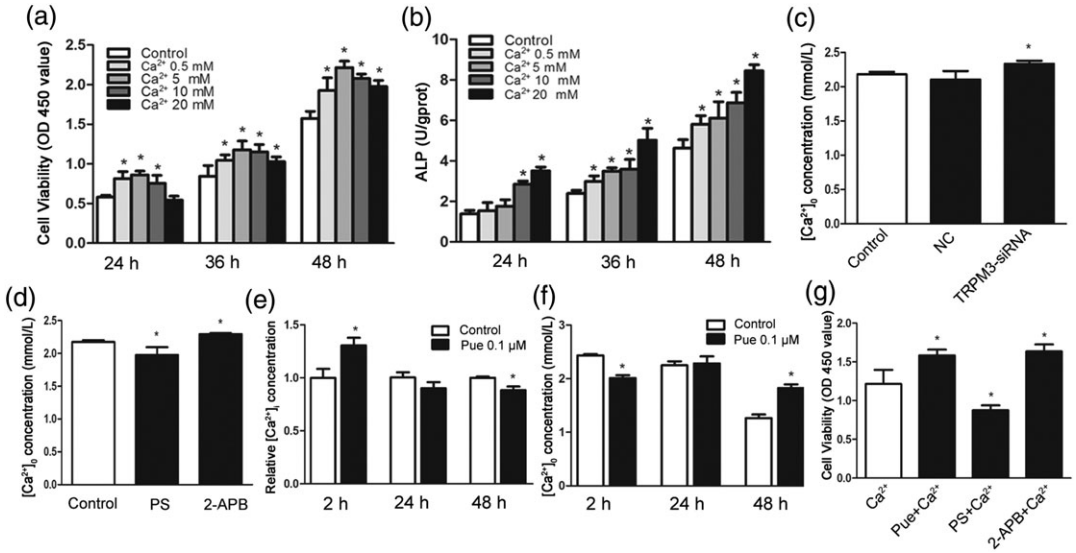

FIGURE 5 Effect of Ca^{2+} on proliferation, differentiation and mineralization in MC3T3-E1 cells. (a) Cells were treated with Ca^{2+} at various concentrations (0.5, 5, 10, 20 μM) for 24, 36, and 48 hr. The cell viability was assessed by CCK-8 assay. (b) Alkaline phosphatase (ALP) activity of the cells was tested after treated with 0.5, 5, 10, 20 μM Ca^{2+} for 24, 36, and 48 hr. (c,d) $[Ca^{2+}]_0$ was measured after transient receptor potential Melastatin 3 (TRPM3) siRNA transfection or pregnenolone sulfate (PS; 100 μM), 2-aminoethoxydiphenyl borate (2-APB; 75 μM) treatment for 48 hr. (e,f) $[Ca^{2+}]_i$ and $[Ca^{2+}]_0$ were measured after treatment with 0.1 μM puerarin for 2, 24, and 48 hr. (g) Cells were pretreated with Ca^{2+} (5 μM) for 30 min and incubated with or without puerarin (0.1 μM), PS (100 μM) and 2-APB (75 μM) for 48 hr. The cell viability was assessed by CCK-8 assay. $^*p < .05$, compared with control

During osteoblast mineralization phase, bone morphogenetic protein, osteocalcin, osteonectin, and other proteins are secreted into the extracellular matrix, following by combining with calcium and phosphorus, which is subsequently mineralized. It has been reported that puerarin could increase the expression of these proteins (An et al., 2016; Chen, Chen, Qi, & Huang, 2016). Obviously, the change of $[Ca^{2+}]_i$ and $[Ca^{2+}]_0$ had an effect on the mineralization of osteoblasts. After 2 hr action of puerarin, extracellular Ca^{2+} entered into cells, resulting in the increase of $[Ca^{2+}]_i$ and promoted osteoblast proliferation and differentiation. After 48 hr, the expression of TRPM3 was decreased and correspondingly reduced the infiltration of Ca^{2+} and lead to the decrease of $[Ca^{2+}]_i$, while the $[Ca^{2+}]_0$ increased. It is possible that the increase of $[Ca^{2+}]_0$ may be prepared for mineralization by combining with the extracellular matrix protein. In addition, the decrease of $[Ca^{2+}]_i$ may be intended to inhibit cell apoptosis that caused by excessive high $[Ca^{2+}]_i$ (Cao, Liu, Zhang, Sun, & Li, 2016). To address this possibility, further studies that investigate the changes of $[Ca^{2+}]_i$ and $[Ca^{2+}]_0$ during mineralization phase are needed.

In conclusion, we show that puerarin promoted MC3T3-E1 cells proliferation, differentiation and mineralization. Following treatment with puerarin, the expression levels of TRPM3 and miR-204 was decreased, which was contrary to that of Runx2, whose expression level was increased. Moreover, the current study demonstrated that puerarin induced the changes of $[Ca^{2+}]_i$ and $[Ca^{2+}]_0$ through TRPM3 might be involved in the promoting effects in MC3T3-E1 cells. The results suggested that puerarin inhibited TRPM3/miR-204 to promote MC3T3-E1 cells proliferation, differentiation and mineralization. These findings may provide a better understanding of the biological effects of puerarin on bone formation in vitro and suggest the potential use of puerarin in prevention and treatment of osteoporosis.

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CONFLICT OF INTEREST

The authors have no conflicts of interests.

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