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Cyanidin-3-glucoside Regulates Osteoblast Differentiation via the ERK1/2 Signaling Pathway

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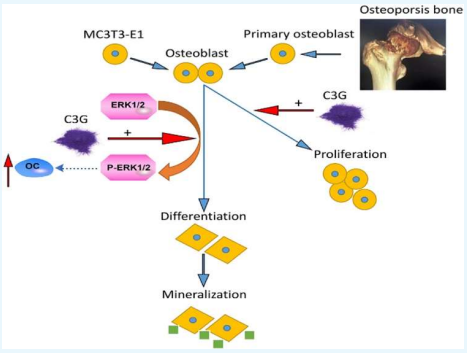
ABSTRACT: Osteoporosis, characterized by a gradual decrease in the number of osteoblasts and a gradual increase in bone resorption of osteoclasts in bone tissue, is a global chronic disease, which severely impairs the quality of life of the elderly. Therefore, it is extremely urgent to study the prevention and treatment of osteoporosis. It has been reported that anthocyanins can regulate bone metabolism and prevent osteoporosis. Cyanidin-3-*O*-glucoside (C3G), the most common type of anthocyanin in nature, widely exists in a variety of vegetables and fruits. Although it has been shown that C3G has multiple effects on osteoclasts, its impact(s) and underlying mechanism(s) on osteoblasts are still not clear. Here, we evaluated the effect of C3G on cell proliferation and differentiation of osteoblasts (extracted from the hip joint of patients with osteoporosis) and MC3T3-E1 (a kind of osteoblast cell line from mice). We also test the ability of osteoblasts to mineralize after C3G treatment. To find the underlying mechanism of the above effects, we further evaluated the role of the ERK signaling pathway in C3G regulation of osteoblasts. The results showed that C3G treatment enhanced osteoblast proliferation rate, osteoblast mineralization points, the mRNA levels and protein expression levels of OC (osteocalcin), and the level of ERK phosphorylation, which could be blocked by pretreatment with ERK signaling pathway inhibitor. The above results not only indicate that the ERK pathway was involved in C3G regulation of osteoblast differentiation but also provide strong suggestive evidence that osteoblasts may be promising targets in preventive and therapeutic strategies for osteoporosis.



INTRODUCTION

Due to its prevalence worldwide, osteoporosis is considered a serious public health concern. Currently, it is estimated that over 200 million people worldwide suffer from this disease. Osteoporosis becomes more common with age. There are 8.9 million fractures worldwide per year due to osteoporosis. This places a large economic burden on the healthcare system due to treatment cost, long-term disability, and loss of productivity in the working population. Therefore, effective prevention of osteoporosis means a great significance. The underlying mechanism in all cases of osteoporosis is an imbalance between bone resorption and bone formation. Osteoclasts degrade the bone matrix, while osteoblasts rebuild the bone matrix. In the whole process of osteoporosis development, inflammatory factors and oxidative stress can inhibit osteogenic synthesis of osteoblasts, thereby aggravating bone loss. Therefore, inhibition of inflammation and oxidative stress to promote osteoblasts rebuilding the bone matrix is very important for the prevention of osteoporosis. Numerous studies have shown that anthocyanins are common flavonoids in vegetables and fruits and have strong anti-inflammatory and antioxidant effects.[1,2] Animal experiments have shown that anthocyanins added to the feed can protect the bones of rats, improve bone mineral content in adult rats, and resist bone loss caused by ovariectomy.[3,4] According to the crowd survey, daily consumption of anthocyanin-rich fruits and vegetables has the effect of preventing bone mineral loss.[5]

At present, there are various hypotheses about the mechanism of anthocyanins against osteoporosis.[3,6−8] However, there are many research studies on anthocyanin regulation of osteoclasts, whereas the effects of anthocyanins on osteoblasts remain unclear. Studies have shown that a variety of anthocyanins can promote the differentiation of mesenchymal stem cells into osteoblasts or improve cell proliferation.[4,9] However, according to the study of Moriwaki et al.,[10] blackcurrant anthocyanins had no significant effect on osteoblasts, and the regulation of anthocyanin was only observed in osteoclasts. The study by Casati et al.[11] demonstrates that delphinidin-3-*O*-rutinoside exerts anti-oxidative damage in osteoblast cell line MC3T3-E1 and

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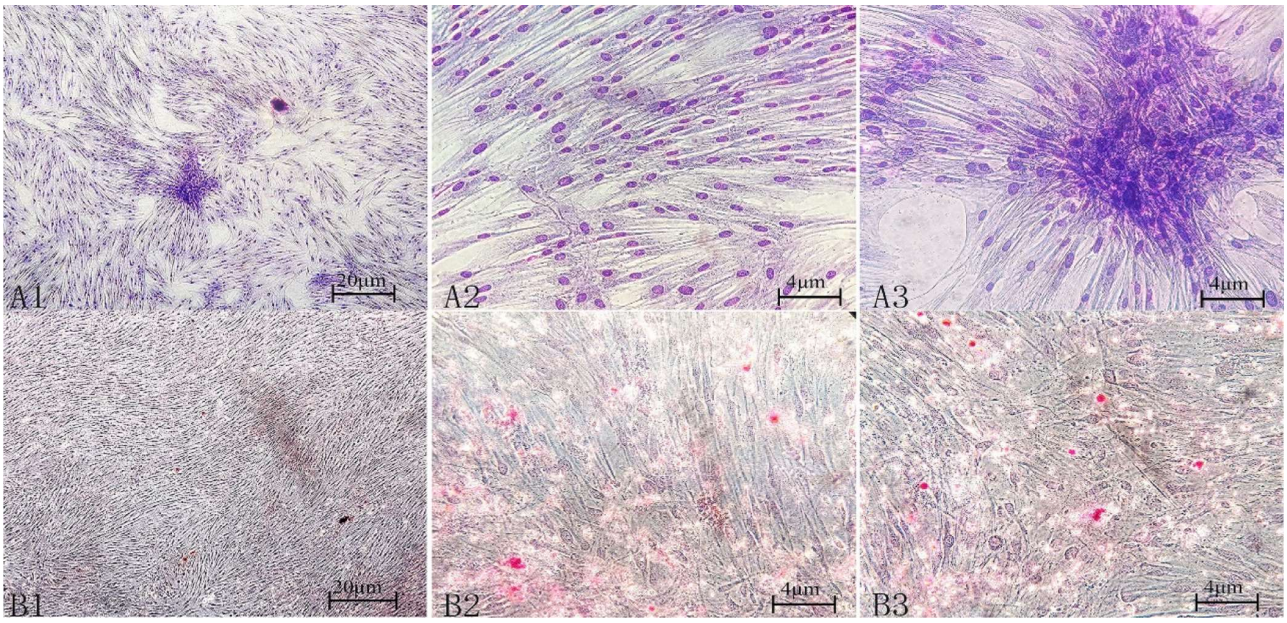

Figure 1. Wright–Giemsa staining and alizarin red staining of human primary osteoblasts. (A1) Wright–Giemsa staining (40×); (A2,A3) Wright–Giemsa staining (200×); (B1) alizarin red staining (40×); and (B2,B3) alizarin red staining (200×).

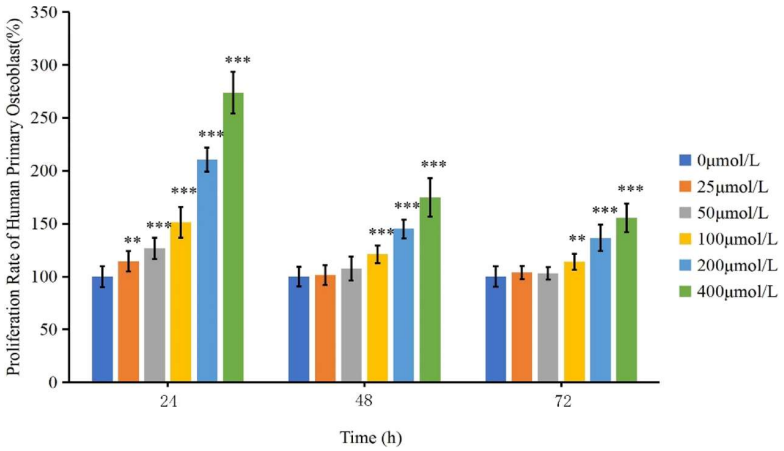

Figure 2. Effect of C3G treatment on the proliferation of osteoblasts. MTT assay of cell proliferation rate difference between various concentration groups at various time points after treatment of human primary osteoblasts with C3G (n = 10).

regulates osteoblast differentiation and proliferation. Park et al.[7] tell us that cyanidin-3-*O*-glucoside (C3G) has regulatory effects on osteoblasts and osteoclasts. C3G is one of the most widely occurring anthocyanins in nature and could quench active oxygen with high efficiency. Taken together, the potential regulation and functional mechanism of C3G on osteoblasts are still unclear.

In the osteoblasts, extracellular signal-regulated kinase 1/2 (ERK1/2), a critical member of mitogen-activated protein kinase (MAPK) cascades, plays an important role in cell proliferation and differentiation. ERK is a positive regulator for osteoblast differentiation and bone formation.[12] Studies have shown that C3G may act through ERK signaling pathways in various cells. Jiang et al. demonstrated that C3G mediated the activation of p-ERK, p-JNK, and p53, which are related to the protection of Sertoli cells and spermatogenesis.[13] C3G in three Thai purple rice cultivars inhibits IL-1β-induced matrix metalloproteinase expression in human articular chondrocytes through the ERK/MAPK pathway.[14] Our previous studies have shown that C3G can promote the proliferation of mouse osteoblast-derived osteoblast cell line MC3T3-E1 in vitro.[15] Thus, we evaluated the role of the ERK signaling pathway in C3G regulation of osteoblasts.

In this study, we used osteoblasts, extracted from the femoral head and neck of patients with hip fractures, as a model to observe the effect of C3G on osteoblasts in vitro. MC3T3-E1 cells, with more stable behavior and characteristics than primary cells, were used to validate the results, as well as the subsequent mechanism studies.

RESULTS

Cell Culture and Identification of Human Primary Osteoblasts. Osteoblasts can be obtained from the cancellous bone of the femoral head by the method described above. After culturing the primary osteoblasts for 24 h, the adherent cells could be seen and were fusiform after changing the solution. In

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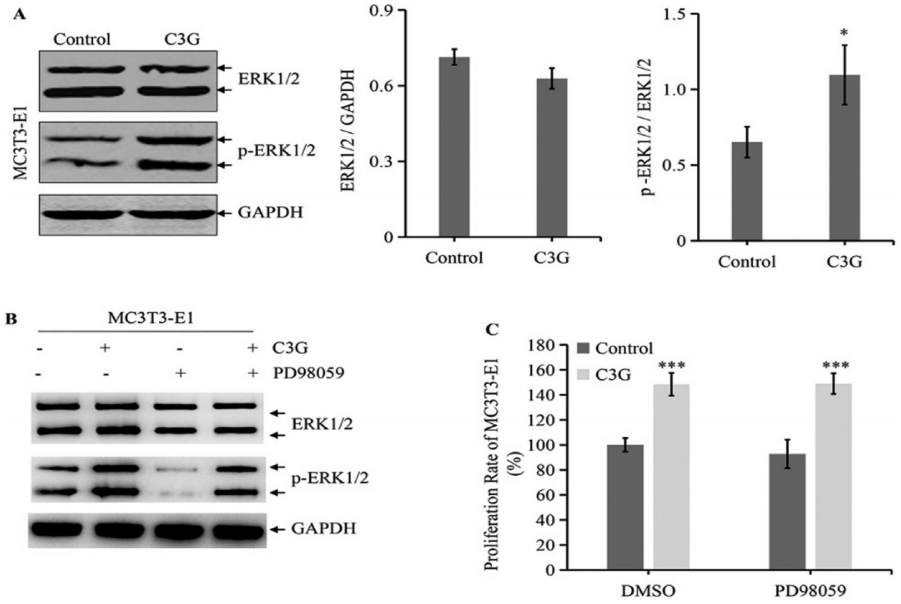

Figure 3. Effect of C3G on osteoblast proliferation after inhibitor pretreatment. (A) Western blot was performed to detect the effect of C3G treatment on ERK1/2 in MC3T3-E1 cells; *$p < 0.05$; comparison with the control group; (B) compared with the C3G group, the C3G + ERK1/2 inhibitor group significantly inhibited the p-ERK1/2 protein expression level. (C) Effect of C3G on proliferation of MC3T3-E1 cells after inhibitor pretreatment; ***$p < 0.001$; comparison with the control group that received the same pretreatment conditions.

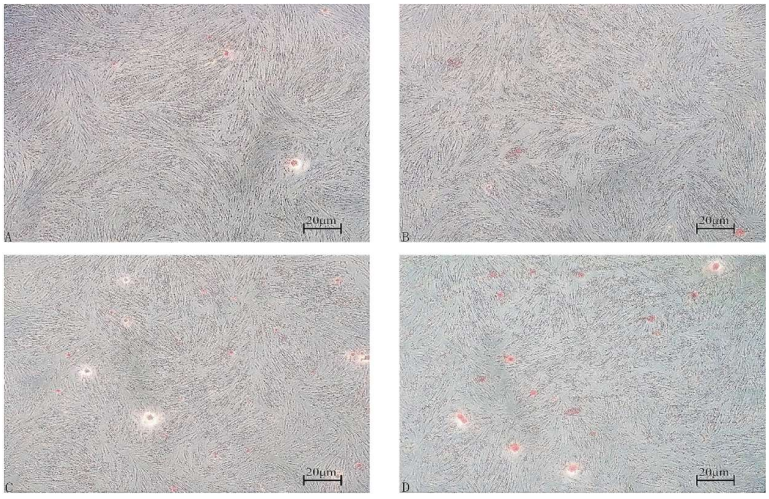

Figure 4. Formation of mineralization points of human primary osteoblasts after various concentrations of C3G treatment ($n = 3$). (A) C3G concentration 0 μmol/L (40×); (B) C3G concentration 50 μmol/L (40×); (C) C3G concentration 100 μmol/L (40×); and (D) C3G concentration 200 μmol/L (40×).

some areas, osteoblast cell−cell overlap could be observed, and it took about 7−10 days for cells to cover the bottom of the flask. After passage, the time required for cell adhesion was lesser than that of the primary cells, and the proliferation rate was more stable. It took only 7 days for the passage cells to fill the bottom of the same size flask, and the shape of cells was long fusiform and polygonal. As shown in Figure 1A, Wright−Giemsa staining results showed that the primary osteoblasts grew adherently and underwent "turbo-like" growth at low magnification (Figure 1A1), characterized by long fusiform, polygonal or stellate shape, rich in cytoplasm, and intracellular mononuclear behavior (Figure 1A2) and showed the tendency to grow in stratified areas (Figure 1A3) at high magnification. After 28 days of induced mineralization, alizarin red staining allowed us to observe approximately elliptical red-stained structures of varying sizes, namely, osteoblast calcification points (Figure 1B1,B2). Primary osteoblasts lacked the ability to pass unlimitedly, and even the cells with the highest ability also completely stopped growing after five to six passages. The above behaviors and characteristics are consistent with that of typical human osteoblasts.

C3G Treatment Promoted Cell Proliferation of Human Primary Osteoblasts. As shown in Figure 2, the MTT results showed that the proliferation rate of human primary osteoblasts significantly increased after C3G treatment compared with the vehicle control group ($p < 0.05$). With the prolongation of treatment time, the difference between the C3G treatment group and the vehicle control group gradually

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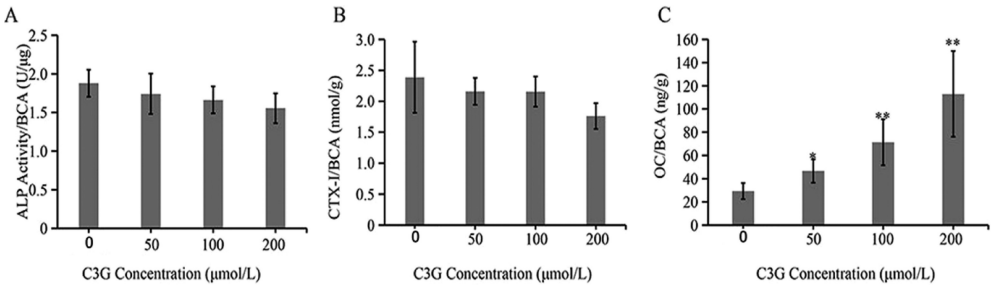

Figure 5. Effect of C3G treatment on ALP activity or OC and CTX-I levels in MC3T3-E1 cells. (A) Activity level of ALP in MC3T3-E1 cells after various concentrations of C3G treatment (n = 4); (B) CTX-I levels in MC3T3-E1 cells after various concentrations of C3G treatment (n = 4); and (C) OC levels in MC3T3-E1 cells after various concentrations of C3G treatment (n = 4); *p < 0.05; **p < 0.01; compared with the 0 μmol/L C3G group.

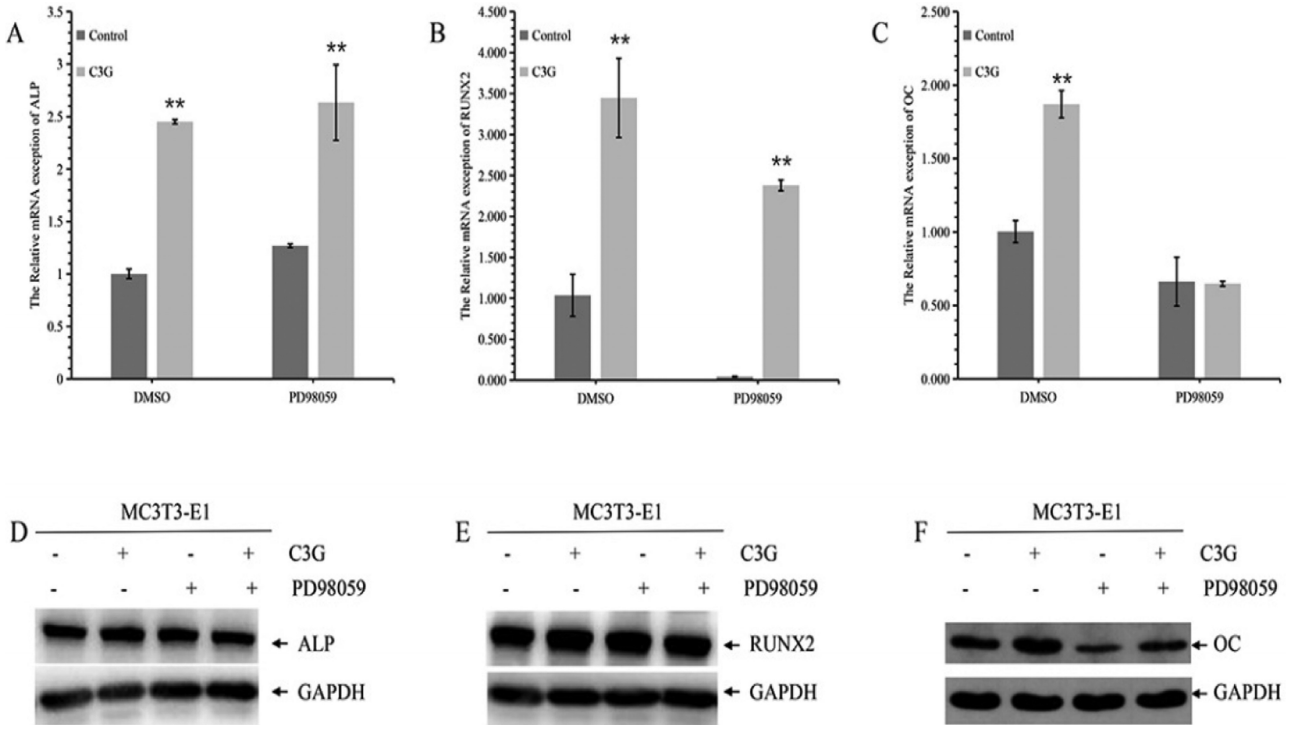

Figure 6. Effect of 100 μmol/L of C3G treatment on ALP, Runx2, or OC mRNA and protein expression in MC3T3-E1 cells with or without PDF98059. (A) ALP mRNA levels in MC3T3-E1 cells were significantly increased in group treated with C3G compared with the control group (FC = 1.55, *p < 0.05, n = 3). After PD98059 pretreatment, ALP mRNA levels increased (FC = 1.34, *p < 0.05, n = 3). (B) Runx2 mRNA levels in MC3T3-E1 cells were significantly increased in group treated with C3G compared with the control group (FC = 1.45, *p < 0.05, n = 3). After PD98059 pretreatment, ALP mRNA levels increased (FC = 2.84, *p < 0.01, n = 3). (C) OC mRNA levels in MC3T3-E1 cells were significantly increased in group treated with C3G compared with the control group (FC = 1.73, *p < 0.05, n = 3). There was no significant difference in OC mRNA levels between the C3G group compared and the inhibitor-control group after PD98059 pretreatment (FC = 0.99, p = 0.93, n = 3). (D) Compared with the C3G group, the ALP protein expression level was not inhibited in the C3G + PD98059 group. (E) Compared with the C3G group, the Runx2 protein expression level was not inhibited in the C3G + PD98059 group. (F) Compared with the C3G group, the OC protein expression level was significantly inhibited in the C3G + PD98059 group.

narrowed. At a time point of 72 h, the difference between 25 and 50 μmol/L of the C3G-treated group and the vehicle control group disappeared (p > 0.05), but 200 and 400 μmol/L of C3G still had a certain enhancement effect on the proliferation of osteoblasts (p < 0.05).

C3G-Activated ERK Pathway Might Not Be Involved in C3G Promoting Osteoblast Proliferation. It has been reported that ERK, as one of a best-studied groups of the MAPK family, appears to promote osteoclast differentiation.[16] Therefore, we evaluate the effect of C3G on total protein expression and phosphorylation of ERK1/2. As shown in Figure 3A, from the results of western blotting, 100 μmol/L of C3G treatment did not affect the expression of ERK1/2 total protein but significantly increased the level of intracellular pERK1/2. PD98059 is an inhibitor for MEK. As shown in Figure 3B, compared with the C3G group, the C3G + ERK1/2 inhibitor group significantly inhibited the p-ERK1/2 protein expression level. We test whether this inhibitor could attenuate the effect of C3G on cell proliferation in osteoblasts. As shown in Figure 3C, the cell proliferation rate was significantly increased in the DMSO-C3G group compared with the DMSO-control group (p < 0.001). After treatment with

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PD98059, the cell proliferation rate of each inhibitor-C3G group was significantly higher than that of the inhibitor-control group ($p < 0.001$). Pretreatment with the ERK inhibitor could not attenuate the promoted effect of C3G on osteoblast proliferation, suggesting that the ERK pathway may not participate in C3G increasing osteoblast proliferation.

C3G Treatment Increased the Mineralization Ability of Human Primary Osteoblasts. As shown in Figure 4, the density of mineralization nodules after treatment of osteoblasts for a week at various concentrations of C3G is shown in the photographs. Human primary osteoblasts were seeded in 12-well plates at a magnification of 40× in the microscope and positioned in the center of the well. The highest C3G treatment concentration (200 μmol/L) produced more red-stained calcifications compared to the control group. A moderate concentration (100 μmol/L) and a low concentration (50 μmol/L) of C3G treatment could also be observed to increase the number of mineralization nodules. These results indicated that C3G treatment increased ex vivo human primary osteoblast mineralization in a dose-dependent manner.

Effect of C3G Treatment on ALP Activity or OC and CTX-I Levels in MC3T3-E1 Cells. After treatment with various concentrations of C3G for 24 h, the intracellular ALP activity and the CTX-I level decreased slightly with the increase of C3G concentration, but the differences were not statistically significant (Figure 5A,B). Compared with the control group, the concentration of OC in each group treated with C3G was significantly increased (Figure 5C).

Roles of ERK1/2 in C3G-Induced mRNA and Protein Expression of ALP, Runx2, or OC in MC3T3-E1 Cells. After treatment with 100 μmol/L of C3G for 24 h, the intracellular ALP, Runx2, and OC mRNA levels and protein levels were significantly increased compared with the control group (Figure 6A−C). After PD98059 pretreatment, there was no significant difference in OC mRNA expression levels between the C3G group and the inhibitor-control group (Figure 6C). Compared with the C3G group, the C3G + ERK1/2 inhibitor group significantly inhibited the OC protein expression level (Figure 6F). Compared with the C3G group, the ALP and Runx2 protein expression levels were not inhibited in the C3G + PD98059 group (Figure 6D,E).

DISCUSSION

Anthocyanins, a class of polyphenolic flavonoids, are pigments found in plant structures that consist of an anthocyanidin (aglycone) attached to sugar moieties.[17] Cyanidin, peonidin, pelargonidin, malvidin, delphinidin, and petunidin are the six major anthocyanidins commonly found in fruits and vegetables.[17] Anthocyanidins are demonstrated to possess health benefits useful for preventing and/or treating chronic diseases.[18] It is known that a high anthocyanin intake is associated with a lower risk of osteoporosis.[19] The addition of anthocyanins to feeds can reverse the tendency of osteopenia in ovariectomized rats or mouse models.[3,20] Sakaki et al. report that blackcurrant supplementation improves trabecular bone mass in young but not aged mice.[21] Anthocyanin composition can vary between different sources and varieties of foods. Thus, anthocyanins' protection and mechanisms of action on bone are likely complex and require further elucidation. There are many research studies on anthocyanins regulation of osteoclasts.[22] However, to date, the mechanism of anthocyanins on osteoblast is still not clear. Casati et al. found that delphinidin-3-rutinoside could increase the proliferation rate of MC3T3-E1 cells, whereas there are no similar studies of C3G on MC3T3-E1 cell proliferation.[11] In previous studies, we had found that C3G has a role in promoting the proliferation of MC3T3-E1 cells.[15] In this study, we used human primary osteoblasts extracted from the femoral head to verify the role of C3G in promoting osteoblast proliferation and found that C3G treatment indeed markedly promoted the proliferation rate of human primary osteoblasts. The human primary osteoblast model is excellent for bone health-related research; the behavior and characteristics of this kind of model are more like that in vivo compared to MC3T3-E1.

In the report by Casati et al.,[11] the optimum concentration of delphinidin for promoting cell proliferation was 0.001 μmol/L, and a higher concentration of delphinidin anthocyanins showed an effect of inhibiting cell growth. However, the results of this study showed that the optimal concentration of C3G was higher than 100 μmol/L, and growth inhibition was not observed at the highest concentration (400 μmol/L). These results indicated that the effective concentrations and effects of different types of anthocyanins on regulating osteoblast function are different. The MTT results of this study showed that the cell proliferation rate of the C3G-treated group decreased when the culture time was too long. We speculated that the possible reason for this phenomenon was that cell density-independent inhibition gradually occurred with the extension of culture time, accompanied by the nutrient consumption in the medium and C3G degradation in the medium. C3G has strong antioxidant capacity but poor stability, so it can be decomposed in the body at a rapid rate, and its degradation rate in a natural environment is faster than that of any other anthocyanins, depending on the type and quantity of functional groups bound by the flavonoid skeleton.[23] We found that there are different effect characteristics of C3G and delphinidin. This is one of the critical findings of this study.

In this study, the samples used to extract primary osteoblasts are challenging to obtain, and cells from different patients lack the essential stability in behavior and characteristics. Therefore, we still use MC3T3-E1 cells as a research model in subsequent mechanism studies (except for cell mineralization experiments). In this study, the expression of specific proteins in the early stage of differentiation and late differentiation of osteoblasts was determined. ALP is expressed in the early stage of osteogenic differentiation and is one of the earliest markers of osteoblast differentiation.[24] In the middle and late stages of osteoblast differentiation, ALP and Runx2 were down-regulated.[25] In the current study, the ALP activity level in MC3T3-E1 cells was low, and there was no significant difference between the C3G-treated groups and the vehicle control groups. The intracellular mRNA levels of ALP and Runx2 were significantly increased in each group treated with C3G compared with the control group. OC accounts for 25% of the total non-collagen bone protein in bone tissue and is one of the most abundant non-collagen bone proteins in bone tissue.[15] The role of OC is to maintain the normal rate of bone mineralization, avoid abnormal mineralization, and promote calcium deposition in bone tissue, and it is an important protein expressed in the middle and late stages of osteoblast differentiation.[26] OC has a hormone-like effect in human body, and its synthesis relies on vitamin D or vitamin K pathway, which can regulate bone mineralization and calcium homeostasis and affect the metabolism of fat and glucose.[27−29] The results of this study fully confirmed that C3G could promote

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OC expression in osteoblasts, which meant that C3G did have a regulatory effect on osteoblast differentiation. Osteoblasts in the late stage of differentiation can produce osteoid and mineralized deposits. Studies related to osteoblasts often use alizarin red or similar dyes to stain mineralized nodules produced by osteoblasts by assessing mineralization capacity. The osteogenic function of osteoblasts was evaluated. Both et al. stained osteoblast-mineralized nodules using alizarin red dye in a study of hydroxychloroquine, inhibiting osteoblast mineralization.[30] Abdallah et al. evaluated the phospholipase D on the mineralization of osteoblasts.[31] The alizarin red staining method was also used to evaluate the differentiation and osteogenic ability of osteoblasts. In this study, after 7 days of induced mineralization, alizarin red staining confirmed that C3G has a positive regulatory effect on the mineralization ability of osteoblasts, and C3G treatment can increase mineralization in cell slides in a dose-dependent manner. This study is based on the above experimental results and other scholars' research report on the stability of C3G, using a regimen of changing every 48 h to balance the effects of C3G degradation and calcification nodule loss caused by fluid exchange.[32]

Osteoblasts experience three phases: cell proliferation, cell differentiation, and extracellular matrix mineralization. Numerous signaling pathways are involved in the osteoblast process, containing the Wnt/β-catenin, ERK1/2, and phosphatidylinositide-3 kinase (PI3K)/Akt pathways.[33] In previous studies, we had found that C3G promotes the proliferation of MC3T3-E1 without Wnt/β-catenin.[15] In this study, it was illustrated that the signaling pathways ERK1/2 had some relationships with C3G's capacity for inducing osteoblast differentiation. MAPK is the main signal molecule in the process of signal transduction and plays an important role in many cell physiological and pathological processes. ERK is one of the MAPK signal pathways. ERK MAPKs have two isoforms, ERK1 and ERK2, both of which are highly expressed in osteoblast-lineage cells. ERK1/2 is activated by phosphorylation.[34] Several studies have shown that the ERK1/2 pathway promotes osteoblast differentiation and bone formation in vitro and in vivo.[12,34,35] The expression of OC is regulated by the ERK1/2 signaling pathway.[36] PD98059 is an inhibitor for MEK, an upstream phosphokinase of ERK1/2. The effect of C3G on cell proliferation was not blocked by this inhibitor. Such a result suggested that the mechanism behind the role of C3G in promoting osteoblast proliferation might be independent of the ERK1/2 pathway. The difference in OC mRNA expression levels between the C3G group and the control group disappeared after pretreatment with this inhibitor. Subsequent western blot results confirmed that C3G treatment increased the phosphorylation level of the ERK1/2 pathway. The above results indicate that C3G upregulates OC expression in an ERK1/2 pathway-dependent manner and regulates osteoblast differentiation. The MAPK signaling pathway has a similar phenomenon in various phosphokinases, and its phosphorylation level has negative feedback regulation on the expression of the enzyme protein itself, which has been found that various anti-tumor drug resistances related to MAPKs pathway is related to this mechanism.[37−39] Based on the above results, C3G can regulate the proliferation and differentiation of osteoblasts under experimental conditions, and the latter effect is related to the ERK1/2 pathway.

CONCLUSIONS

This study found that C3G can promote the proliferation of human primary osteoblasts in vitro; in addition, C3G treatment down-regulated the expression levels of some marker genes in the early stage of osteoblast differentiation, and the expression level of osteocalcin, working in late stage, is improved. In addition, C3G can increase the mineralization capacity of osteoblasts, and these regulatory effects related to osteoblast differentiation may be achieved through the ERK1/2 signaling pathway.

METHODS

Primary Cell Isolation and Cell Culture. Human primary osteoblasts were extracted from femoral heads that were removed during surgery from patients undergoing femoral head replacement after the femoral neck fracture. The connective tissue at the end of the femoral head was dissected in a sterile environment, and then the portion of the cancellous bone was cut from the sample using a rongeur. The cancellous bone specimen was completely cut in a Petri dish using ophthalmic scissors, and after washing, it was digested with type II collagenase (Sangon, China) to obtain a primary human osteoblast sample. The cells obtained by the above operation will be inoculated into a culture flask. When these cells grew to cover 80% of the bottom of the bottle, the cells were trypsinized and passaged. After induction of mineralization, alizarin red was used to stain potential mineralization nodules for the identification of osteoblasts, and then third generation cells were used for subsequent experiments. Primary human osteoblasts and MC3T3-E1 cells for subsequent studies were both cultured in an incubator containing 5% concentration of carbon dioxide using alpha modification minimum essential medium (α-MEM, HyClone, USA) containing 10% standard fetal bovine serum (HyClone, USA).

Cell Proliferation. MTT (Jiancheng, China) assays would be used to measure the proliferation of cells. Adjust the cell density to 4×10^4/mL and inoculate the cell suspension into a 96-well plate at 100 μL per well. After the cells were attached, the cells were synchronized by removing the serum in the medium for 24 h. In some experiments, 4 h before the end of the synchronization treatment, a concentration of 50 μmol/L of PD98059 (Selleck, China) or an equal concentration of DMSO (Sangon, China) was added to the wells for inhibition of the MEK enzyme site. After the completion of the synchronization treatment, the medium in the wells was changed to a complete medium containing various concentrations of C3G, and incubation was continued for 24, 48, or 72 h. The MTT test is done according to the instructions in the kit.

Induction of Osteoblast Mineralization and Alizarin Red Staining. Alizarin red dye can stain the mineralization nodules produced by osteoblasts, which could be used to identify isolated primary cells or to evaluate the effect of C3G on the mineralization ability of osteoblasts. The cell density was adjusted to 7×10^5/mL, then inoculated into a Petri dish having a diameter of 60 mm, and the inoculum amount per dish was 1 mL. In the study for the identification of osteoblasts, the cells were cultured for 14 days, and then the medium in the dish was changed to a complete medium containing 10 mmol/L of β-glycerophosphate. The treatments were continued for 14 days, and the sample was stained

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according to the method of using the alizarin red dye. In an assay for evaluating the mineralization capacity of C3G on osteoblasts, cells were seeded in 12-well plates. The density of the seeded cells was 1×10^4/mL, and the inoculum amount was 1 mL per well. Primary osteoblasts were cultured for 7 days in a complete medium containing various concentrations of C3G, changing every 2 days because of the poor stability of C3G. After 7 days, the medium in the wells was removed, and after the cells were fixed as necessary, they would be stained with alizarin red dye. The appearance of calcified nodules was examined under an inverted microscope and photographed and stored.

Alkaline Phosphatase Activity Assay. The MC3T3-E1 cell density was adjusted to 7×10^5/mL, and the cells were seeded in the 100 mm diameter Petri dish, and the inoculum amount was 2 mL per dish. After waiting for the cells to adhere, the cells were synchronized for 24 h by using a serum-free medium. After the completion of the synchronization treatment, the medium was replaced with a complete medium containing various concentrations of C3G, and the culture was continued for 24 h. After the end of the C3G treatment, the cells were washed with PBS and then collected by a cell scraper. By disrupting the cell sample by sonication, the total protein content of the sample will be quantified; then, the alkaline phosphatase kit will then be used to determine the activity of alkaline phosphatase in the sample. The alkaline phosphatase activity data measured for each sample would be normalized by the previously measured protein concentration results.

Determination of Osteocalcin and Carboxy-Terminal Collagen Crosslink Expression Level. The MC3T3-E1 cell samples were prepared using the same treatment as the alkaline phosphatase activity assay. The expression levels of OC and CTX-I (carboxy-terminal collagen crosslinks) in the sample were determined by ELISA. The results will be normalized by the total protein concentration results for each sample.

Real-Time PCR. The MC3T3-E1 cell samples would be treated in a similar manner to the alkaline phosphatase activity assay or added to PD98059 for inhibition of the MEK enzyme site. Samples would be digested with Trizol (Takara, Japan), then total RNA was extracted with chloroform and precipitated with isopropanol. The assay was carried out using Takara's cDNA synthesis kit (Takara, Japan) and qPCR kit (Takara, Japan). All primers used in this study are as follows: ALP-F 5′-CACGGCGTCCAT GAGCAGAAC-3′ and ALP-R 5′-CAGG-CACAGTGGTCAAGGTTGG-3′, Runx2-F 5′-GCAGCAG-CAGCAGCAGAG-3′ and Runx2-R 5′-GCACGGAGCACAG-GAAGTTG G-3′, OC-F 5′-AGACTCCGGCGCTAC CTTGG-3′ and OC-R 5′-CGGTCTTCAAGCCA-TACTGGTC TG-3′. The method of data analysis is $\Delta\Delta CT$.

Western Blot. The MC3T3-E1 cell samples would be treated in a similar manner to the alkaline phosphatase activity assay or added to PD98059 for inhibition of the MEK enzyme site. The protein in the sample collected by the cell scraper would be extracted by the total protein extraction kit, which would be separated by electrophoresis on polyacrylamide gel. Later, the separated protein would be transferred to the PVDF membrane and then blocked with skimmed milk. After incubation with the primary antibody (anti-ERK1/2, Immunoway, USA; anti-Perk1/2, Cell Signaling Technology, USA; anti-OC, ABclonal, USA; anti-ALP, Abcam, USA; anti-RUNX2, Abcam, USA; and anti-GAPDH, Abcam, USA) and the secondary antibody (anti-Rabbit, Immunoway, USA), imaging was taken under the action of the developer.

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Author Contributions

B.Z. designed the study; B.H., L.C., Z.Z., and X.W. developed the methodology; Y.C. contributed the reagents and materials; B.H. and B.Z. contributed to data analysis and interpretation; B.H. and B.Z. wrote and reviewed the manuscript; and B.Z. and B.H. involved in funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Notes

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ABBREVIATIONS

ALP, alkaline phosphatase; C3G, cyanidin-3-glucoside; CTX-I, carboxy-terminal collagen crosslinks; OC, osteocalcin; RUNX2, runt-related transcription factor 2

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