Pro-bone and Antifat Effects of Green Tea and Its Polyphenol, Epigallocatechin, in Rat Mesenchymal Stem Cells in Vitro

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ABSTRACT: Green tea has been demonstrated recently as a potent bone supportive agent. Our previous studies showed that green tea and its polyphenolic constituents can promote bone-forming osteoblast activities and inhibit the bone-resorbing osteoclast formation. The objective of the present study was to investigate whether green tea and its components can regulate the osteogenic and adipogenic differentiation in pluripotent rat mesenchymal stem cells (MSCs). The rat MSCs were isolated from the bone marrow of tibiae and femora. The cells were treated with decaffeinated green tea extract (GTE) and six tea polyphenols under osteogenic induction. The alkaline phosphatase (ALP) activities and matrix calcium (Ca) deposition were assessed after 7 and 14 days of treatment. Our results demonstrated that GTE could significantly increase ALP dose dependently in the concentrations without cytotoxicity ($0-100$ μg/mL). Among six tested tea polyphenols, epigallocatechin (EGC) was shown to be the most effective in promoting osteogenic differentiation. At 20 μM, EGC increased ALP levels and Ca deposition significantly by 2.3- and 1.7-fold, respectively, when compared with the control group. EGC also increased the mRNA expression of bone formation markers runt-related transcription factor 2, ALP, osteonectin, and osteopontin. Furthermore, EGC demonstrated its antiadipogenicity by decreasing the adipocyte formation and inhibiting the mRNA expression levels of the adipogenic markers peroxisome proliferator-activated receptor γ, ccaat/enhancer-binding protein β, and fatty acid binding protein 4. In conclusion, this is the first report of the dual action of green tea polyphenol EGC in promoting osteogenesis and inhibiting adipocyte formation in MSCs. Our results provide scientific evidence to support the potential use of green tea in supporting the bone against degenerative diseases such as osteoporosis.

KEYWORDS: bone metabolism, green tea, epigallocatechin, osteogenesis, adipogenesis, mesenchymal stem cell

■ INTRODUCTION

Green tea is one of the most extensively studied herbs with well-regarded health benefits, a long history of consumption, and wide safety margins. Common green tea polyphenols include (−)-epigallocatechin-3-gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin-3-gallate (ECG), and (−)-epicatechin (EC).[1] These polyphenols have been shown to be epimerized to (−)-catechin (C), (−)-gallocatechin (GC), (−)-catechin gallate (CG), and (−)-gallocatechin gallate (GCG), respectively, after heat treatment.[2] EGCG accounts for more than 50% of the total green tea ingredients, followed by EGC and ECG.[3] These compounds have beneficial effects in preventing cancers,[4] cardiovascular diseases,[5] and neurodegenerative conditions.[6]

Recently, a number of epidemiological studies have been conducted on the beneficial effect of tea consumption on bone health.[7,8] These epidemiological studies reported that the bone mineral density of postmenopausal women who have a habit of tea drinking was higher than that of other women. Our previous in vitro studies illustrated that EGC could increase the bone formation activities in UMR-106 osteoblast-like osteosarcoma cells and inhibit the osteoclast differentiation from murine monocyte/macrophage RAW 264.7 cells.[9] EGCG, another important green tea polyphenol, also possessed similar biological activities in increasing the formation of mineralized bone nodules in human osteoblast-like, SaOS-2 cells[10] and inducing

the apoptotic cell death of osteoclasts differentiated from RAW 264.7 cells in vitro.[11] These findings suggested that green tea may improve osteoporotic conditions by inhibiting the progressive bone loss due to both increased osteoclastic bone resorption and decreased osteoblastic bone formation.

Mesenchymal stem cells (MSCs) are adult pluripotent progenitor cells that give rise to osteoblasts, adipocytes, chondrocytes, and myocytes. There is a reciprocal relationship between the differentiation of adipocytes and osteoblasts.[12] Clinical observations have shown an increase in differentiation of MSCs into adipocytes rather than osteoblasts in a variety of osteoporosis.[13] Therefore, the enhancement of osteogenesis with a concomitant decrease in adipogenesis could provide a novel therapeutic target to the treatment of osteoporosis, to increase bone formation by diverting the adipogenesis in MSCs to osteogenesis.[14] Reports of the bone-protective effects of green tea and its polyphenols in cell lines and animals have highlighted this herb as a candidate for the prevention of osteoporosis. Moreover, Furuyashiki et al. reported that green tea polyphenols suppressed adipocyte differentiation in the preadipocyte 3T3-L1 cell line.[15] There has been

speculation that green tea and its polyphenols can promote osteogenesis and suppress adipogenesis in primary pluripotent MSCs. However, this assumption has not yet been fully proved. Therefore, in our present study, we aim to investigate whether green tea extract (GTE) and its components can regulate the differentiation of MSCs derived from the rat in vitro.

■ MATERIALS AND METHODS

Chemicals. C, EC, GC, EGC, GCG, and EGCG were from Sigma Chemical Co. (St. Louis, MO). A standard stock mixture of C, EC, GC, EGC, GCG, and EGCG at 20 mM each was prepared in dimethyl sulfoxide (DMSO) and stored in small aliquots at −80 °C until use. Decaffeinated GTE, Sunphenon DCF-1, was a gift from Taiyo Kagaku Co. Ltd. (Tokyo, Japan). Each 166 mg of GTE contained 53.6 mg of EGCG, 24.2 mg of GCG, 21.6 mg of EC, 17.6 mg of EGC, 17.0 mg of GC, and 7.2 mg of C. All other chemicals were purchased from Sigma unless other specific.

Culture and Characterization of Rat MSCs. Rat MSCs were cultured from the bone of the tibiae and femora of male Sprague−Dawley rats (250 g) by centrifugation as described previously.[16] Isolated bone marrow cells were resuspended in growth medium [α-MEM (Life Technologies, United States) containing 10% fetal bovine serum and 1% penicillin/streptomycin (Life Technologies)] and seeded at a density of $2 \times 10^5/cm^2$ at 37 °C in 95% humidified air and 5% CO_2. On day 7, all nonadherent cells were removed and followed by a medium change twice a week. The monolayer of adherent cells was trypsinized by 0.25% trypsin EDTA when it reached half-confluence and reseeded at a density of $1 \times 10^4/cm^2$ [passage 1 (P1)]. P2 culture was used for all in vitro assays. The identity of the culture was characterized by flow cytometry using the CANTO ll with the FACs Diva software (BD Biosciences, United States), which showed positive results in two MSCs cell surface molecules, CD44 and CD90, and negative results in two hematopoietic markers, CD11b and CD45.

Differentiation Protocol and Drug Treatment. For the differentiation studies, P2MSCs were seeded in six-well plates at a density of 2×10^4 cells/cm². After 3 days, the growth medium was replaced with the osteogenic medium (growth medium supplemented with 100 nM dexamethasone, 50 μg/mL ascorbate-2-phosphate, and 10 mM β-glycerol phosphate) or the adipogenic medium (growth medium supplemented with 1 μM dexamethasone, 50 μg/mL insulin, 0.5 mM methyl-isobutylxanthine, and 100 μM indomethacin), respectively, with a medium change twice a week. GTE was prepared as a stock solution in either osteogenic or adipogenic medium and sterilized by filtration with 0.22 μm filter. This was then added to both differentiation media to provide final concentrations in the range within 100 μg/mL. All studied tea polyphenols would be screened at 20 μM. These dosages of GTE and polyphenols were previously established to produce no cytotoxic effects on MSCs throughout the differentiation experiments. Genistein (20 μM) was used as a positive control agent for osteogenesis and a negative control agent for adipogenesis,[17] whereas troglitazone (2 μM) was used as a positive control agent for adipogenesis.[18]

Assessment of Osteogenic Differentiation Markers. To determine the effects of GTE and its components on osteogenesis in MSCs, the related biochemical markers alkaline phosphatase (ALP) activity and matrix calcium deposition were measured according to manufacturer protocols. The ALP activity was measured in the cell culture using the commercial available ALP activity kit (Stanbio, United States) after 7 days of osteogenic treatment. In brief, each sample was rinsed twice with PBS and extracted with lysis buffer consisting of 50 mM Tris-HCl (pH 7.2), 0.1% Triton X-100, and 2 mM $MgCl_2$. The lysate was then subjected to two freeze−thaw cycles at −70 °C. The supernatant was collected after centrifugation at 10000g for 10 min, and ALP activities were determined in assay buffer containing

p-nitrophenylphosphate. The optical intensity of the reaction mixture was measured at 405 nm after 15 min of incubation. The total protein content was determined using bicinchoninic acid protein assay reagent, and enzyme activities were expressed as U/mg protein. The matrix calcium deposition was quantified using StanbioTotal LiquiColor calcium determination kit (Stanbio) after 14 days of osteogenic treatment, and the readout was normalized by protein content.

Assessment of Adipogenic Differentiation Markers. The number of adipocytes was determined using Oil Red O staining method.[17] After 21 days of EGC treatment in adipogenic medium, the cultured cells were rinsed twice with PBS and fixed with 10% buffered formalin (v/v) for 10 min. Fixed cells were washed and stained with 0.2% Oil Red O in 60% isopropanol for 15 min. Excessive stain was removed by one change of 60% isopropanol and then distilled water three times. Photomicrographs were taken with an inverted microscope at 100× magnification. The number of adipocytes per field was calculated by averaging the counting Oil Red O positive cells in eight separated fields. The cell viability of undifferentiated MSCs was also determined by the 3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyl-tetrazolium bromide (MTT; Sigma, United States) assay after 21 days of treatment with EGC at various concentrations in 96-well plates (5×10^2 cells/well). The relative cell viability was determined by measuring the reduction of MTT dye in metabolically active cells to blue formazan crystals at an optical density of 540 nm and expressed as the percentage of control group without EGC treatment.

Real-Time Polymerase Chain Reaction (PCR) on Osteogenic and Adipogenic mRNA Expression. After 7 days of EGC treatment in both osteogenic and adipogenic medium, the total mRNA of each treated cell culture was isolated by the RNeasy mini kit (Qiagen, GmbH) and stored at −20 °C. The amount of mRNA was determined using the Quanti-Fast SYBR Green RT-PCR kit (Qiagen) with a validated primer set from Qiagen specific for osteogenic markers [runt-related transcription factor 2 (Runx2; NM_053470), ALP (NM_013059), osteonectin (NM_012656), and osteopontin (NM_012881)] and adipogenic markers [peroxisome proliferator-activated receptor γ (PPAR-γ; NM_013124), CCAAT/enhancer binding protein β (C/EBP-β; NM_024125), fatty acid binding protein 4 (Fabp4; NM_053365), and adipsin (XM_343169) mRNA expression on the CFX96 Real-Time PCR Detection System (Bio-Rad)]. The threshold cycle (Ct), the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was determined. Relative expression of the RT-PCR product was calculated using the comparative $2^{-\Delta\Delta Ct}$ method. The endogenous control glyceraldehyde-3-phosphate dehydrogenase mRNA was used for normalization. The fold difference was then determined by normalizing all values to the mean of the relative expression for the control group without treatment.

Statistical Analysis. The differences between treatment and control groups were tested with the Kruskal−Wallis test, followed by the posthoc Dunn's test. All statistical analyses were performed by using the Statistical Package of Social Science (SPSS) version 15.0 for Windows and carried out at the 5% level of significance ($P < 0.05$). Data are expressed as means ± standard deviations (SDs).

■ RESULTS

Characterization of Rat MSCs. Rat MSCs were isolated from primary bone marrow cells, and a homogeneous fibroblastic layer was formed (Figure 1). As there is no single specific cell marker for MSCs, a panel of markers for flow cytometry was chosen. The P2 rat MSCs culture was consistently expressed for CD44 (homing-associate cell adhesion molecules, Figure 1a) and CD90 (Thy-1, Figure 1b) but was negative for CD45 (leukocyte common antigen, Figure 1a) and CD11b (monocyte/macrophage lineage marker, Figure 1b).

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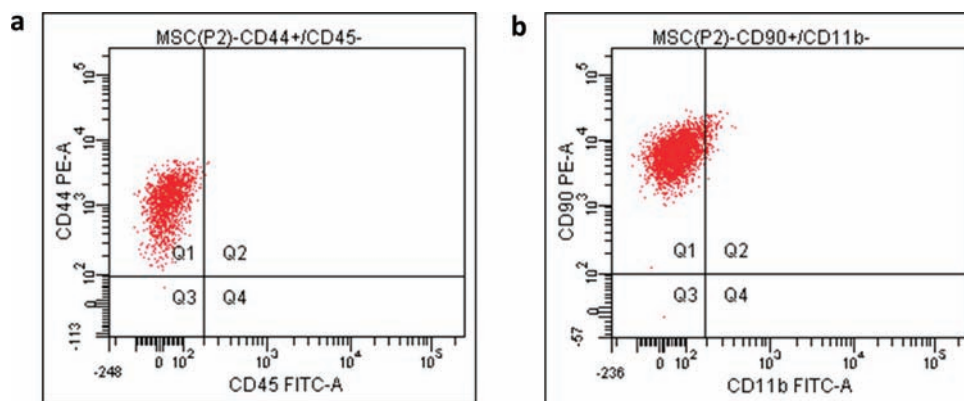
Figure 1. Rat MSCs surface markers were assessed using flow cytometry. Fluorescence intensity dotplots with specific antibodies (Ab). Phenotypes of MSCs labeled with Ab against PE-CD44 (with FITC-CD45 negative; a) and PE-CD90 (with FITC-CD11b negative; b). All experiments were performed in triplicate.
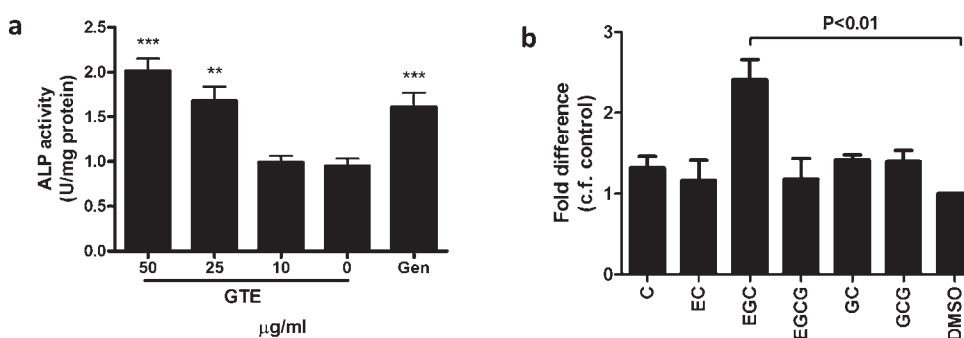
Figure 2. (a) Dose effect of GTE on alkaline phosphatase (ALP) activity in MSCs were determined under osteogenic induction on day 7. (b) Effect of different green tea polyphenols (20 μM) on ALP activity in MSCs on day 7 were also determined. Genistein (Gen, 20 μM) acted as a positive control. Data are the means ± SDs (n = 3) from three independent experiments. Significant difference: *P < 0.05, **P < 0.01, and ***P < 0.001 for difference from respective baseline cultures without treatment.
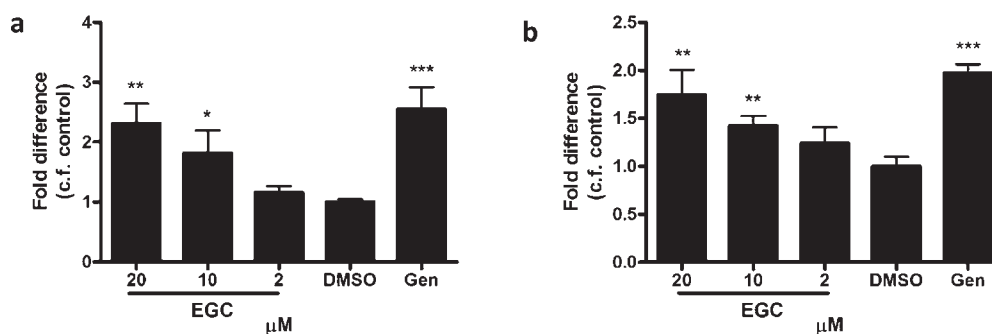

Figure 3. Osteogenic properties of EGC in rat MSCs. Dose effect of EGC on (a) alkaline phosphatase (ALP) activity and (b) extracellular matrix calcium content in MSCs were determined under osteogenic induction on days 7 and 14, respectively. Genistein (Gen, 20 μM) acted as a positive control. Data are the means ± SDs (n = 3) from three independent experiments. Significant difference: *P < 0.05, **P < 0.01, and ***P < 0.001 for difference from respective baseline cultures without treatment.

Effects of Green Tea and Tea Polyphenols on Osteogenic Differentiation of Rat MSCs. To determine whether tea could stimulate osteogenic differentiation, the effect of GTE on bone formation marker, ALP activity, was studied. Our data illustrated that treatment of MSCs with GTE for 7 days stimulated ALP activity in a dose-dependent manner (Figure 2a). At 50 μg/mL, the ALP activity was significantly increased by about 2-fold when compared with the respective control without treatment.

Its osteogenic effect was similar to the positive control genistein at 20 μM.

To find out the active tea polyphenol(s) responsible for osteogenic action of tea, six tea polyphenols, C, EC, EGC, EGCG, GC, and GCG, were tested. As shown in Figure 2b, there was a significant increase in ALP activity upon EGC treatment at 20 μM. However, the addition of other tea polyphenols at the same concentration had no effect on ALP activity.
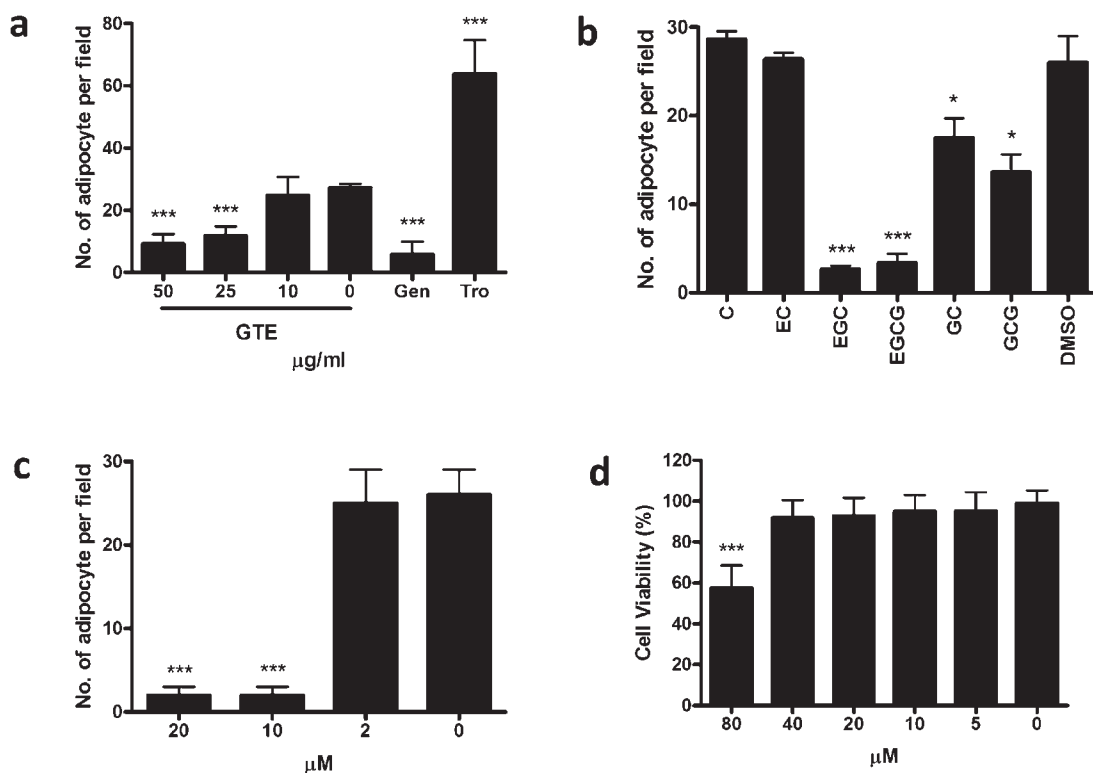
Figure 4. (a) Inhibitory effect of GTE in MSCs under adipogenic induction on day 21. The number of adipocytes was counted after oil-red O staining. Genistein (Gen, $20\,\mu$M) was used as an inhibitory control, and troglitazone (Tro, $2\,\mu$M) was used as a positive control. (b) Effect of different green tea polyphenols ($10\,\mu$M) and (c) dose effect of EGC against adipogenesis in MSCs on day 21 were also determined. (d) Effect of EGC on the cell viability of P2 undifferentiated MSCs. MSCs were treated with different concentrations of EGC for 21 days. Data are the means $\pm$ SDs ($n = 3$) from three independent experiments. Significant difference: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ for difference from respective baseline cultures without treatment.

We further investigated the osteogenic effect of EGC in MSCs. As shown in Figure 3a, the ALP activity was increased in a dose-dependent manner after 7 days of induction. The effect of EGC in osteogenic differentiation as evidence by extracellular matrix mineralization was also investigated. At $20\,\mu$M, EGC significantly increased the matrix calcium deposition by 72% ($P = 0.003$). A dose-dependent response of calcium deposition was also observed at lower concentrations of EGC.

To further confirm the osteogenic potential of EGC, the mRNA expressions of bone formation markers, Runx2, ALP, osteonectin, and osteopontin were determined with quantitative real-time PCR in the cells after 7 days of treatment. As shown in Figure 5a, EGC treatment significantly increased the gene expression of Runx2, ALP, osteonectin, and osteopontin at $20\,\mu$M when compared with control vehicle DMSO group. For the positive control group genistein, it significantly increased the gene expression of Runx2, osteonectin, and osteopontin, but not ALP, which suggests that the action mechanisms by EGC and genistein may be different in promoting the osteogenic differentiation of MSCs.

Effects of EGC on Adipogenic Differentiation of MSCs. To investigate the effect of GTE on adipogenic differentiation in rat MSCs, Oil Red O staining was used to visualize the formation of adipocytes after 21 days of adipogenic induction. As shown in Figure 4a, GTE significantly inhibited the formation of adipocytes in a dose-dependent manner. At $50\,\mu$g/mL of GTE, the number of adipocyte was decreased by 66.3% ($P < 0.001$). Its effect was similar to the positive control genistein in decreasing

the number of adipocyte by about 78% ($P < 0.001$). For positive control troglitazone, the number of adipocyte was increased by 135% ($P < 0.001$) on day 21.

Among six tested polyphenols, EGC, EGCG, GC, and GCG were shown to be effective in decreasing the adipocytes formation at $10\,\mu$M (Figure 4b). Similar antiadipogenic effects were found in both EGC and EGCG. Both of them exert stronger effects than GC and GCG in antiadipogenesis. However, the addition of other tea polyphenols at the same concentration had no effect on adipocyte formation. For EGC, a dose-dependent antiadipogenic response was observed in the concentration range from 0 to $20\,\mu$M (Figure 4c). At $20\,\mu$M, EGC significantly decreased the adipocyte number by 91.3% ($P < 0.001$). Our data also demonstrated that that EGC did not affect the cell viability in a range of $0-40\,\mu$M on day 21, suggesting that EGC inhibited adipogenesis without affecting the cell number (Figure 4d). In contrast, extensive cell death was found when the cells were treated with EGCG at $20\,\mu$M in adipogenic medium for 21 days (data not shown). The adipocytes may undergo either apoptosis or necrosis.

To further confirm the antiadipogenic potential of EGC, the mRNA expressions of adipocyte formation markers, C/EBP-β, PPAR-γ, Fabp4, and adipsin were determined with quantitative real-time PCR in the cells after 7 days of EGC treatment. Because the treatment period is relatively short, we expect EGC could regulate the early transcriptional adipogenic markers such as C/EBP-β and PPAR-γ instead of the terminal marker adipsin. As shown in Figure 5b, EGC significantly inhibited the gene

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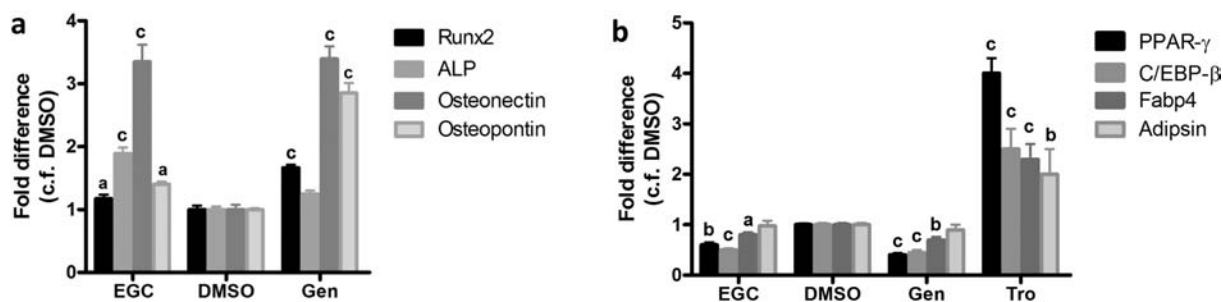
Figure 5. Effect of EGC on mRNA expression of osteogenic and adipogenic markers in rat MSCs under osteogenic or adipogenic induction. MSCs were treated with 20 μM EGC for 7 days. Genistein (Gen, 20 μM) and troglitazone (Tro, 2 μM) were used as positive controls in both osteogenic and adipogenic differentiation, respectively. Genistein was also used as an inhibitory control of adipogenesis. The mRNA expression levels of Runx2, ALP, osteonectin, osteopontin, PPAR-γ, C/EBP-β, Fabp4, and adipsin were determined using real-time PCR. The expression levels of both genes were normalized on the basis of GAPDH expression. Fold differences were determined as the relative expression, as compared with control cells with vehicle DMSO treatment. Data are the means $\pm$ SDs (n = 3) from three independent experiments. Significant difference: a, $P < 0.05$; b, $P < 0.01$; and c, $P < 0.001$ for difference in mRNA expression from culture with DMSO treatment.

expression of PPAR-γ (0.6 $\pm$ 0.05-fold), C/EBP-β (0.5 $\pm$ 0.03-fold), and Fabp4 (0.8 $\pm$ 0.05-fold) at 20 μM. This pattern was similar to the inhibitory control genistein in decreasing both adipogenic regulator mRNA expressions. In contrast, for the positive control group treated with PPAR-γ agonist troglitazone at 2 μM, it significantly increased the gene expression of PPAR-γ (4.0 $\pm$ 0.3-fold, $P < 0.001$), C/EBP-β (2.5 $\pm$ 0.4-fold, $P < 0.001$), Fabp4 (2.3 $\pm$ 0.3-fold, $P < 0.001$), and adipsin (2.0 $\pm$ 0.5-fold, $P = 0.004$) when compared with control vehicle DMSO group.

■ DISCUSSION

Apart from the elevation of osteoclastogenesis, there has been accumulating evidence suggested that the imbalance between osteogenesis and adipogenesis in bone marrow cell differentiation, with adipogenesis predominant over osteogenesis, is another key factor of osteoporosis in older adults.[19] An inverse relationship between increasing marrow fat and decreasing bone mineral density was found.[14] This accumulation of marrow fat may simply represent a passive process with fat reoccupying the vacated space by trabecular bone or exhibiting a more active process with marrow fat intensively accumulating at the expense of bone centered on a change in mesenchymal cell differentiation from an osteoblastic to an adipocytic pathway.[20] This leads to the suggestion that osteoporosis may be a type of lypotoxic disease with osteoporosis being the "obesity of bone".[21]

In our present study, we showed that GTE and one of the tea polyphenols, EGC, significantly increased the bone formation in osteoprogenitor rat MSCs. This was indicated by the dose-dependent increase in bone marker ALP activities, upon the addition of GTE and EGC to the MSCs in osteogenic medium. In contrast, no similar change was observed when the other tea polyphenols were added to the culture. While it is true that on day 7 EGC increased ALP activity, it should be noted that the peak ALP activity can be reached whenever between day 7 and day 14, and it is possible that other polyphenols were not treated long enough to attain the peak effect. The increase of ALP activity enhances the hydrolysis of phosphate-containing substrates into orthophosphate and initiated the calcification of the bone.[22] The osteogenic activities of EGC were further confirmed by the increase in calcium deposition in extracellular matrix and elevation in bone markers: Runx2, ALP, osteonectin, and osteopontin mRNA synthesis. Runx2 is a key transcription factor required for in vivo bone formation and osteoblast differentiation.[23]

It is expressed in mesenchymal condensation of developing bones during embryogenesis, and the mRNA was shown to increase in osteogenic tissues. Osteonectin is a glycoprotein that binds calcium and is secreted by osteoblasts during bone formation, thus initiating mineralization and promoting mineral crystal formation.[24] Osteopontin is an acidic phosphoprotein, which is associated with cell attachment, proliferation, and mineralization of extracellular matrix into bone.[25]

Recently, there were contradictory reports showing the other forms of tea polyphenol involve in both osteogenesis and adipogenesis.[26,27] For instance, EGCG could increase the expressions of osteogenic genes, elevate ALP activity, and eventually stimulate mineralization in a murine bone marrow MSC line, D1.[27] However, in our study, EGCG did not possess the pro-bone effect in rat MSCs. This may be due to the fact that MSCs originating from different species (or strains) may possess different osteogenic potential and act in their own way in response to exogenous stimulus. Besides, skeletal development is highly orchestrated by genetic background.[28] Peister et al. reported that the differentiation potentials of five strains of inbred mice were quite different.[29] MSCs from Bl/6, DBA1, and GFPtg mice differentiated more readily into osteoblasts than adipocytes, but BALB/c and FVB/N MSCs differentiated more readily into adipocytes.

Apart from osteogenesis, we found that EGC dose dependently inhibited the adipocyte formation in MSCs as well. This observation was further substantiated by the mRNA expression of the inhibition of adipogenesis transcription factor, C/EBP-β, and adipocyte marker, Fabp4. PPAR-γ and C/EBP-β are essential transcriptional factors for adipocyte differentiation and maturation. In vivo studies illustrated that mice with a deletion in the gene for PPAR-γ had lower levels of blood triglycerides, free fatty acids, cholesterol, and hepatic triglyceride accumulation.[30] Similar findings were also demonstrated in C/EBP-β knockout mice.[31] It was reported that C/EBP-β regulated the expression of PPAR-γ during adipogenesis via MEK/ERK signaling pathway.[32] Lipid accumulation within adipocytes is promoted by the gene encoding FABP4, which is under the transcriptional control of PPAR-γ.[33] FABP4 has a high affinity for a variety of fatty acids and facilitates their storage, trafficking, and solubilization.[34] Therefore, FABP4 has been used as a marker to follow the differentiation of adipocytes. However, EGC only caused a slightly but statistically insignificant decrease in adipsin

mRNA expression. This may due to the fact that the experimental period was not long enough for MSCs to differentiate completely to produce and express mature adipocyte markers.

With regard to aging, accumulative oxidative damage and weakened antioxidative defense systems cause a disturbance in the organism's redox balance[35] and lead to age-related osteoporosis.[36] In this context, green tea polyphenols act as a good source of antioxidant to work against oxidative damage. Shen et al. reported that green tea polyphenols mitigated bone loss in ovariectomized middle-aged female rats via an increased antioxidant capacity and a decreased oxidative stress damage.[37] They found that green tea polyphenols mixture also reduced bone loss in a chronic inflammation-induced bone loss model, probably by reducing oxidative stress-induced damage and inflammation.[38] The antiobesity and osteoprotection of green tea polyphenols may potentially protect the bone in high-fat diet-induced obese rats.[39] Recently, it was also found that oxidative stress induced apoptosis, senescence, and inhibited osteoblastic differentiation in human MSC in vitro.[40] Green tea polyphenols, particularly EGC, may take its action in promoting bone formation not only by its direct stimulatory osteogenic effect but also together with its antioxidative abilities in MSCs.

To interpret the clinical significance of our in vitro findings, the bioavailability of EGC in human subjects would be an important consideration. Lee et al. showed that the plasma levels of EGC in healthy volunteers increased to 223 ng/mL after drinking brewed green tea (1.2 g/200 mL hot water).[41] This suggested that drinking more than 25 cups of green tea is required to attain the effective in vitro dose of 20 μM. As such tea consumption is not so practical, the administration of appropriate green tea polyphenols in concentrated form may be achieved to give beneficial effects to the bones. Besides, the oral bioavailability of tea polyphenols may be enhanced when consumed in the absence of food. This pharmacokinetic data are important for the design of future clinical trial to exploit the full potential of green tea polyphenols in osteoporosis management.

Our study has provided the first laboratory evidence on the pro-bone and antifat effects of the green tea and EGC, which was demonstrated by the promotion of osteogenic and inhibition of adipogenic differentiation in rat MSCs. However, the mechanism of action of EGC in bone protection still remains to be elucidated. Studies could be achieved by exploring signaling pathways upon treatment of EGC in MSCs. Furthermore, the beneficial effects of tea will be further investigated in human MSCs. The pro-osteogenic and antiadipogenic data from human cell should be included to consolidate our findings. As the routine consumption dose is far lower than the effective one, it is absolutely imperative to test the efficacy of the concentrated form of polyphenol in an animal model, prior to proceeding to a human study.

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■ **ABBREVIATIONS USED**

EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin-3-gallate; GC, (−)-gallocatechin; GCG, (−)-gallocatechin gallate; MSCs, mesenchymal stem cells

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